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Ethanol Tolerance and Dependence: Endocrinological Aspects

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
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Research Monograph No. 13

Ethanol Tolerance and Dependence: Endocrinological Aspects

PROCEEDINGS OF A WORKSHOP
September 14–15, 1982

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The Editor: Theodore J. Cicero, Ph.D., is the Biomedical Scientific Director, Alcohol Research Center of the Washington University School of Medicine.

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Foreword

This monograph reports on the proceedings of a research symposium held in September 1982 and cosponsored by the Alcohol Research Center of the Washington University School of Medicine and the National Institute on Alcohol Abuse and Alcoholism (NIAAA). The Alcohol Research Center is one of eight centers funded by NIAAA. Each center focuses on a central theme of importance to the study of alcoholism, ranging from the neuropharmacological effects of alcohol consumption to the epidemiological aspects of the production, distribution, and consumption of alcoholic beverages.

In addition to the primary mission of developing new knowledge through original research in their chosen areas of interest, the centers are responsible for disseminating that knowledge to the scientific and lay communities. In this connection, the centers have joined NIAAA in organizing and holding workshops and symposia on topics in their specialty areas. The proceedings in this volume represent one of these collaborative activities.

The purpose of the Washington University symposium was to examine a number of issues related to the effects of alcohol on endocrine hormones. Several decades ago, theories were advanced regarding the role of endocrine system dysfunction as a cause of alcoholism. Such theories were based on similarities between the symptoms seen in alcoholic patients and in patients with some endocrine disorders. At that time, research evidence did not provide support for a causal role; it appeared the available information suggested that the endocrine characteristics associated with alcoholism were a result of chronic heavy drinking rather than its cause.

Over the past four decades, substantial advances have been made in research technologies, and the knowledge base in the two fields of alcoholism and endocrinology has been expanded. It seemed timely, therefore, to bring together researchers working in this area to discuss their most recent findings and to indicate what research should be done next. These proceedings report on their discussions.

It is hoped that this report will increase the awareness of and interest in research on alcohol-endocrine relations and that new research will be stimulated on this aspect of alcohol use and abuse.

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Introduction

Theodore J. Cicero

Ethanol, like most substances of abuse, has significant acute and chronic effects on virtually every endocrine system. Because hormones have such a pervasive influence on the function of all organs in the body, it seems highly probable that ethanol-induced perturbations in specific hormones could contribute to both its acute pharmacological profile and, particularly, to its long-term actions. Since tolerance to and physical dependence on ethanol are characterized by persistent and dramatic functional, biochemical, and physiological changes in many organs, principally the liver and brain, many investigators have postulated that hormones might be uniquely suited for a role in mediating at least some aspects of these phenomena. Recent studies lend much support to this hypothesis, and most of this body of knowledge is contained in the proceedings of this meeting.

Because of the recent surge of interest in ethanol-endocrine interactions, and the possible role of drug-induced endocrine abnormalities in the development of metabolic and functional tolerance to and physical dependence on ethanol, a symposium directed at these issues was considered timely. Accordingly, under the joint auspices of the Alcohol Research Center of the Washington University School of Medicine, and the National Institute on Alcohol Abuse and Alcoholism, a symposium was held at the Washington University School of Medicine on September 14 and 15, 1982.

The Alcohol Research Center of the Washington University School of Medicine was initially funded in 1977 for a 5-year period; it was competitively renewed in December 1982 for an additional period of support. In reality, however, Washington University School of Medicine has had a Program Project or Alcohol Research Center since the late 1960s, and its current status merely represents the reorganization and formalization of national centers that occurred in 1977. Our center is a multidisciplinary one, emphasizing the epidemiological, neurobiological, and genetic aspects of alcoholism. More than 35 senior investigators participate in the center, and their activities are coordinated by the project director (Samuel B. Guze, Spencer T. Olin Professor and head of the department of psychiatry and vice chancellor for medical affairs) and three scientific directors: Theodore J. Cicero (biomedical/neurobiological), Theodore Reich (genetic), and Lee N. Robins (epidemiological).

As program director, I solicited the aid of five distinguished scientists to organize sessions to fully cover the existing data, to provide an overview and historical perspective, and to indicate areas that need further study. As will be evident, these five individuals recruited the preeminent workers in their respective fields to present a comprehensive and excellent overview of this important area of research.

The sessions and their chairpersons were:

(1) Tolerance to and Physical Dependence on Ethanol--General Considerations, A. Eugene LeBlanc, Chairperson

(2) Endocrine Effects of Ethanol, David H. Van Thiel, Chairperson

(3) Role of Hormones in Metabolic Tolerance to Ethanol, Yedy Israel, Chairperson

(4) Role of Hormones in Functional Tolerance to and Physical Dependence on Ethanol, Floyd E. Bloom, Chairperson

(5) Membrane Mechanisms of Ethanol Tolerance and Physical Dependence, Dora B. Goldstein, Chairperson.

The symposium was organized to provide, at the outset, an overview of the methodological and theoretical issues involved in the experimental analysis of ethanol tolerance and physical dependence, since it would be impossible to evaluate studies of hormonal influences on these processes without an appropriate characterization of just what these phenomena represent. Similarly, the full spectrum of ethanol's endocrine effects was examined to emphasize the diverse impact of this drug on the endocrine system. In two subsequent sessions, the available data were critically reviewed concerning the involvement of hormones in both metabolic and functional tolerance to ethanol. In the final session, the membrane mechanisms involved in ethanol tolerance and physical dependence were reviewed. Although such a topic may not seem to mesh well with the main focus of the symposium, this session was considered important for three reasons: (1) quite obviously, hormones interact with membrane-bound receptors; (2) ethanol has significant influences on membrane structure and function; and, (3) much recent data have accumulated to suggest that additional studies of ethanol-membrane interactions may lead to some important new breakthroughs with a significant impact for all ethanol-related research.

The reader will, I believe, find these proceedings interesting, informative, and comprehensive. This state-of-the-art review will not only serve to educate but to stimulate increased research interest in this important area.

TOLERANCE TO AND PHYSICAL DEPENDENCE ON ETHANOL– GENERAL CONSIDERATIONS

A. Eugene LeBlanc, Chairperson
Jatinda M. Khanna
Herbert Barry III
Richard A. Meisch
V. Gene Erwin
Boris Tabakoff

Overview

Jatinda M. Khanna,
A. Eugene LeBlanc,
and Anh Dzung Lê

The purpose of this review is to analyze and discuss briefly the key themes or issues in alcohol tolerance and physical dependence that are currently being pursued and to examine some of the older literature to see how these issues have been treated in the past or how they were foreshadowed by some of the issues that were discussed in earlier works. Our interest is in the evolution of the ideas in alcohol tolerance and physical dependence. Scientific ideas have often been initiated and developed, then forgotten, and later rediscovered. As we proceed, we point out some examples where that certainly was the case.

It might be important to explain why this whimsical approach to reviewing alcohol tolerance has been taken. When one of us (J.M.K.) joined our Toronto alcohol research group, he began his studies on acquired tolerance to alcohol. When a report on this work (Hawkins et al. 1966) was already in an advanced state of preparation, we came across an early report by Pringsheim (1908) that showed that not only had much of the essential issue been examined but also many features of our experimental design had similarities with this work that was done some 60 years earlier. This chance occurrence gave us the idea that perhaps either there were important things to learn from some of the very early literature or at least that credit to the early researchers was warranted.

Another author (A.E.L.) had occasion to participate in a major review on tolerance to, and physical dependence on, alcohol and other nonopiate psychotropic drugs (Kalant et al. 1971), in which the statement was made that "it has been known for centuries that regular drinkers generally become able to tolerate large amounts of ethanol on repeated exposure." Many other workers have made similar statements. In discussing the matter, however, the authors of that review became aware that it would be very difficult to provide a scholarly reference for that assertion.

To respond to this joint interest in reevaluating the contributions of the earlier researchers on alcohol tolerance, we have gathered and examined an assortment of older materials. From the 19th century we reviewed various books on inebriety, including

government publications and treatises. In the work since 1900 we used the Classified Abstract Archive of the Alcohol Literature, prepared by the Journal of Studies on Alcohol, and standard bibliographic materials to find early studies on alcohol tolerance. We also tried to find materials in each decade to the present to sample the material systematically enough for a reasonable perspective.

Alcohol monographs of the 19th century dealt primarily with the ill effects associated with chronic consumption of alcohol. Pathology of the brain and liver, gastronintestinal function, sexual dysfunction, tuberculosis and, in fact, any other possible malady was attributed to alcohol. The deleterious effects of alcohol on the mind, willpower, intellect, and soul were attributed not only to chronic consumption but even to occasional drinking.

Despite the fact that it was generally acknowledged that individuals differ markedly in their ability to handle alcohol, the term "tolerance" was not mentioned in the 19th century. Even the terms "habituation" or "adaptation" were not mentioned. Thus, before the 20th century, no distinction was made between "innate" and "acquired" tolerance, despite the explicit observations reported by many authors of differences in alcohol intoxication among individuals. The terms "hallucination" and "delirium" were, however, regularly described as toxic features of alcohol consumption.

Thus, our investigation of the literature from the 1800s was relatively unilluminating. Although the concept of tolerance was implicit in a few cases and was quite clearly implicit in the case of Anstie (1865), our current review of the 19th century literature did not uncover any explicit analysis of the issues of tolerance. This is all the more perplexing since, as we show later, by the early 20th century quite sophisticated analyses of those issues had already begun.

The work of Anstie (1865) grew out of a concern about alcohol as a food, and there was much debate in the 1800s, often for doctrinaire reasons, about whether alcohol could be considered a food. It is quite clear from the literature that the extent to which alcohol could be considered a food would provide a basis on which the drinking of alcohol could be excused, fostered, or encouraged. As a related concern, Anstie became interested in the capacity of an individual to consume alcohol. In effect, he attempted to define how tolerant an individual could be, or what the individual's capacity to consume alcohol would be, both from the point of view of safety at any time and from the point of view of the total amount of alcohol that could be metabolized within a fixed period. In effect, Anstie was dealing with a form of innate metabolic tolerance. With that exception, we do not deal further with the 19th century.

MECHANISMS OF TOLERANCE

Systematic studies of alcohol tolerance began at the start of the present century. This is clearly reflected in the extensive and thorough studies of Mellanby (1919), which are familiar to most investigators in alcohol research. Mellanby's objective was to study the absorption, distribution, and metabolism of alcohol in various parts of the body and the influence of other factors, such as food, fasting, and type of alcoholic beverage, to explain the variability of alcoholic effects in different individuals. The observations on degree of impairment were made primarily for this purpose rather than to study acquired tolerance.

Despite the fact that Mellanby's work is widely cited, that of his predecessors and contemporaries has been almost totally forgotten today, yet many of their questions and concepts are remarkably current.

For example, a significant amount of research during the past 10 to 15 years has been concerned with the question of whether chronic ethanol treatment results in an increased ability to metabolize ethanol (i.e., metabolic tolerance), and if so, by what mechanism (Kalant et al. 1971). The existence of metabolic tolerance as a result of chronic ethanol treatment was demonstrated convincingly some years ago. However, the mechanism(s) of metabolic tolerance are still being disputed. Many researchers have reviewed and analyzed these studies (for references, see Wallgren and Barry 1970; Lundquist 1971; Hawkins and Kalant 1972; Lieber 1977; Khanna and Israel 1980). One may assume that these analyses represent current rigor in analyzing the problem as a result of the advances in pharmacology and the more sophisticated basis of research today.

This, however, is not the case. Research in the early 20th century shows that metabolic tolerance has been examined thoroughly by various authors, such as Rosenfeld (1901), Pringsheim (1908), Schweisheimer (1913), Gettler and Freireich (1935), Newman and collaborators (Newman and Card 1937a,b), and Levy (1935). Moreover, the original researchers were quite sophisticated in their articulation of the working hypothesis. This is clearly reflected in the review article by Bogen (1936), who considered and discussed in depth the following factors as an explanation for a mechanism of acquired tolerance:

(1) Destruction of alcohol in the gastrointestinal tract before absorption;

(2) Retarded passage of alcohol into the general circulation from the gastrointestinal tract;

(3) Increased oxidation of alcohol in the liver before it reaches general circulation;

(4) Storage of alcohol somewhere in the body--i.e., removal of it from circulation;

(5) Rapid oxidation or destruction of alcohol in the body;

(6) More rapid excretion of alcohol in the expired air, urine, etc.; and

(7) Reduced penetration of alcohol into the brains of habituated animals.

On the basis of his studies and a review of other research, Bogen concluded that "none of the various mechanisms described for the development of tolerance to alcohol may be accepted as a complete explanation for the phenomenon observed." Since at that time an increased cellular resistance to alcohol had not yet been demonstrated, Gettler and Freireich (1935), on the basis of their work and previous research, came out strongly in favor of metabolic tolerance as the sole explanation for alcohol tolerance. In fact these authors, in discussing physiological resistance, went so far as to say, "When no explanation for tolerance to a certain substance is available, then the term acquired tolerance is commonly used as a subterfuge."

Another idea that has been pursued actively in recent years is the issue of metabolic cross-tolerance between ethanol and other drugs. It has recently been shown that chronic administration of ethanol results in an increased metabolism of some agents such as carbon tetrachloride and acetominophen, thereby resulting in an increased hepatotoxicity of these drugs (Hasumura et al. 1974; Teschke et al. 1979; Licht et al. 1980). As early as 1907, Hunt was examining the relationship between alcohol consumption and the toxicity of a number of other substances. His explicit working hypothesis was that chronic exposure to alcohol would increase the metabolic capacity for alcohol (remember that the matter of alcohol tolerance was not clearly settled until the late 1960s) and that increased alcohol metabolism would increase the metabolism of other agents. In fact, in some elegant studies in animals kept on ethanol for 6 to 10 weeks, he showed convincingly that chronic ethanol administration resulted in increased metabolism of acetonitrile. Moreover, he showed that in animals that were fed dextrose, this was not the case.

Another issue that has attracted attention in recent years is the question of alcohol metabolism in relation to its concentration in the body. This issue also was examined extensively by the early workers, and an enormous amount of literature is available on the subject (for references see Carpenter 1940). Researchers who are interested in this topic should carefully and thoroughly read these early studies before embarking on one of their own.

Concerning acquired central nervous system (CNS) tolerance, many investigators have recently asked about the nature of the relationship between "learned tolerance" and "physiological

tolerance." Much research has been done in this area, and it appears that learned tolerance is essentially the same as physiological tolerance, except that the former is acquired more rapidly (Kalant et al. 1971; Kalant and Khanna 1980; Kalant 1982). This problem of whether tolerance is caused by some change in the tissues (i.e., do the nerves become insensitive to the effects of the alcohol) or by the occurrence of some learning by which the effects can be coped with more effectively also confronted early researchers. This is clearly stated by Newman and Card (1937a):

> Another possible explanation for the apparent tolerance to moderate concentrations of alcohol shown by the habituated dogs warrants consideration. It is conceivable that habituation to alcohol is productive of a psychological adjustment to the long-continued presence of this substance in the brain. Thus the habituated individual probably learns to compensate for his lack of coordination and removal of inhibitions, and consequently does not exhibit the same degree of abnormality as does the abstainer who has not learned to make the necessary allowances for the effect of the drug. This might well account for the absence of tolerance which we found for higher, definitely toxic, concentrations. We may draw an analogy with the tabetic, who can be reeducated to walk fairly well, with certainly no increase in functioning nervous tissue.

Similarly Goldberg (1943), in his excellent quantitative studies on alcohol tolerance in humans, suggested psychological compensation as one of the factors that causes greater ethanol resistance.

Early researchers seem to have been handicapped in that they did not use behavioral methods per se but seemed ready to interpret their results or at least consider a behavioral explanation. They were also quite aware of the issue of learning but were unable to resolve it because of deficiencies in methodology rather than an inability to specify clearly the nature of the problem. It is, therefore, not surprising that the rigorous analysis of the role of learning in alcohol tolerance, which has been carried out so thoroughly during the past decade, escaped early researchers.

CROSS-TOLERANCE AND REVERSAL OF TOLERANCE

Another very important issue of current research interest is that of cross-tolerance between alcohol and other drugs such as opiates. Research on this subject has been stimulated by the realization that the traditional pharmacological categories are not absolute. Despite the differences in the drug classes to which

morphine and ethanol belong (opiate-analgesics versus sedative-hypnotics) and the differences in their pharmacological properties, cross-tolerance between morphine and ethanol was recently demonstrated with respect to hypothermia (Khanna et al. 1979; Mansfield et al. 1981) and electrically induced contraction in the isolated guinea-pig ileum longitudinal muscle/myenteric plexus preparation, which is inhibited acutely by both drugs (Mayer et al. 1980).

However, the notion of examining cross-relationships between disparate drugs is not new. In fact, Myers (1916) studied cross-tolerance between morphine and chloral hydrate as well as between morphine and cannabis. He concluded that, "The experiments cited show that a cross-tolerance may exist between closely related drugs but that this tolerance is evidenced only on those functions in which the drugs have a common selective action."

This hypothesis is very similar to that proposed by Kalant and coworkers during the 1970s. They suggest that the stimulus to tolerance is not the drug itself but the functional disturbance that it produces (Kalant et al. 1971; Kalant and Khanna 1980; Kalant 1982).

Another issue that is still being pursued has had a renaissance of interest in recent times--i.e., the reversal of tolerance or the acquisition of sensitivity as a result of chronic exposure to alcohol. In a series of experiments designed to maximize the degree of tolerance produced by ethanol by sustaining high-level exposure for several weeks, we discovered that tolerance began to reverse spontaneously even though the alcohol treatment continued. On the basis of various recent studies (for references, see Khanna et al. 1980a,b), we postulate that while ethanol was producing and maintaining tolerance, it was concurrently undermining the capacity of the brain to sustain tolerance by the production of chemically induced brain lesions.

On reviewing the literature, we found that the phenomenon of tolerance reversal late in an alcoholic's drinking career was described by many authors in almost every one of the past six decades (Medical Research Council 1923; Schweisheimer 1920; Bogen 1936; Peck 1949; Merkel 1959; Rotter 1959; Jellinek 1960; Esser 1963; Pyatnitskaya 1966). Moreover, even the very earliest works reported--but without data and without literature citations--cases in which hypersensitivity to alcohol was acquired as a result of chronic exposure to alcohol. However, we could find no reports of experimental research on this subject. Here is a good example of a topic on which a rereading of the older literature could have provided a stimulus to modern research, especially since we now have techniques that did not exist 60 years ago.

Despite the development of good methods for measuring alcohol effects in experimental animals, researchers today are still concerned with methods of producing tolerance. In fact, during

the past 10 to 15 years various methods have been developed for chronic administration of alcohol and the production of tolerance and dependence in animals (see Cicero 1980). However, as early as 1907, Hunt was administering alcohol to mice, guinea pigs, and rabbits in their diet (Hunt 1907). By using a very narrow-necked feeder tube, he exposed the animals to grains that had been soaked in various concentrations of alcohol. He also recognized that evaporation makes the various dietary techniques subject to the loss of some alcohol during administration. It is also noteworthy that the technique of administration of alcohol by inhalation introduced by Goldstein (1971), which circumvents the problems of evaporation loss and bioavailability, was developed and described in detail by MacLeod (1948) nearly 25 years earlier.

Most researchers in alcohol are familiar with the work of Victor and Adams (1953) and Isbell et al. (1955), who showed beyond doubt that the hyperexcitability phenomena, such as convulsions, hallucinations, and delirium tremens, are caused by the sudden withdrawal of ethanol in alcoholics. As early as 1912, Hare had clearly presented the same concept and captured the essence of the view presented later by Victor and Adams and by Isbell and coworkers. He said (Hare 1912):

> Acquired tolerance implies intolerance of sudden or very rapid withdrawal. The chief complications which are liable to arise are delirium tremens and alcoholic epilepsy, or both. There must be no sudden fall in the amount given, and this is especially important during the first two or three days. On the other hand, after the fourth or fifth day, when the amount of alcohol has fallen to 4 or 5 ounces, further tapering is often necessary. Undoubtedly, the most important point from the standpoint of avoiding symptoms and complications is the cautious reduction from the amount of acquired tolerance during the first three or four days. Failure to recognize this is accountable for many cases of delirium tremens. The author believes that delirium tremens is an alcoholic emergency depending primarily upon acqulred tolerance, and is a direct result of a sudden inadequacy in the regular supply of alcohol for the blood and nervous system. The proximate factor in delirium tremens consists in the too sudden withdrawal of alcohol from the system, and accordingly it is possible to completely abort an attack which is actually commencing by means of prompt and adequate doses of alcohol.

The clarity of Hare's statement and, as we now recognize, the accuracy of his interpretation do not detract from the elegance of the proof put forward by later works. Hare's hypothesis was, after all, an idea waiting for its time. It is just one more example of ideas in the early literature that can serve as points of departure for modern research.

CONCLUSIONS

In many cases the early researchers had quite modern views of the problems that we are now pursuing. Many of the advances in alcohol research have more to do with improved methods and knowledge than with improved working hypotheses. For instance, in recent years researchers have been extensively studying learning factors in tolerance, changes in membrane fluidity, receptor alterations, $Na^+ + K^+$-ATPase changes, and so forth--approaches that represent a sophistication of the fundamental ideas rather than a significant departure from the basic conceptual framework.

It is also apparent that in research on tolerance, as perhaps in other areas of research, facts are discovered, forgotten, and then rediscovered. Thus, a knowledge of the older literature may offer some major advantages:

(1) It may help us to avoid unnecessary repetition of research that has been done before--e.g., metabolic tolerance to ethanol and the effect of concentration on ethanol metabolism.

(2) It can provide ideas to investigate with modern techniques--e.g., the role of learning in tolerance and tolerance as an adaptation to the drug effect rather than to the drug itself.

(3) It may provide clues to ideas that were not fully formulated in the past--e.g., the reversal of tolerance and cross-tolerance.

Finally, a knowledge of the history of our research field provides a culture and a context into which to fit our contributions. This can provide great intellectual satisfaction, a sense of perspective of the developments to which we are contributing, and perhaps a healthy sense of modesty about our own times and our place in science.

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Adaptive Behavior of Alcohol Tolerance and Withdrawal

Herbert Barry III

Behavior is an integrated response of the entire organism to its external and internal environments. An important function of behavior is to make both environments compatible with each other. The intense effect of alcohol on the central nervous system (CNS) is a severe challenge to behavior. Alcohol tolerance is an adaptive response that diminishes the change in the internal environment caused by the drug. Withdrawal illness results from the persistence of the adaptive response when the drug is no longer present.

PURPOSE OF THIS REVIEW

Various types of behavior are used to measure alcohol tolerance and withdrawal. Studies of tolerance and withdrawal were reviewed thoroughly by Wallgren and Barry (1970). Briefer, more recent reviews have been written by Barry (1979) and by contributors to a book edited by Rigter and Crabbe (1980), especially the excellent chapter by Cicero (1980). These reviews include studies in both humans and laboratory animals, but the emphasis is on laboratory animals because the controlled experiments permit more definite conclusions.

Behavior includes responses to the external environment associated with the onset or disappearance of drug effects. A review by Barry (1978) discusses the role of these conditional responses to the external environment in aversive, reinforcing, and discriminative drug effects.

The present review summarizes evidence for the role of conditional responses to the external environment in alcohol tolerance and withdrawal. Compensatory responses that counteract the drug's effect may contribute to tolerance and withdrawal. Hinson and Siegel (1980) have summarized experiments in laboratory animals, demonstrating that these compensatory responses can be induced by the external environment associated with alcohol administration.

BEHAVIORAL RESPONSES TO ALCOHOL EFFECTS

We generally regard our behavior as being directed toward the external environment. Our brain receives messages from outside the body, through the sensory modalities of seeing, hearing, smelling, touching, tasting, and pain. Our skeletal muscles enable the behavioral responses to change the outside world or to change our location in it.

The highly integrated function of behaving also responds to signals from the internal environment. We receive and respond to signals from our body organs. Alcohol and other drugs produce internal changes that induce behavioral responses. These behavioral responses help to maintain the constancy of our internal environment by modifying diverse physiological functions or by adjusting to them.

High doses of alcohol severely depress the CNS (Wallgren and Barry 1970). This pharmacological depression is accompanied by behavioral stimulation. During alcohol intoxication, people gesture vigorously, talk loudly, and sometimes act violently. Laboratory animals under some conditions explore a novel environment more actively. These responses have been attributed to the stimulant effects of low alcohol doses or to disinhibitory effects (Wallgren and Barry 1970).

Another important mechanism for this stimulated behavior may be a compensatory response to counteract the drug's depressant effect. Exposure to a low alcohol dose may be expected to induce such a compensatory response. A mild degree of intoxication is a good predictor of subsequent severe drug effects. A small amount of alcohol is often followed by further consumption or by absorption and distribution of a large amount of alcohol already consumed.

EXTERNAL ENVIRONMENT ASSOCIATED WITH ALCOHOL

Tolerance to alcohol develops when the animal is administered the drug and tested in the external environment that is associated with previous alcohol administrations. When alcohol is given in a different environment, the tolerance disappears.

Table 1 summarizes three experiments that demonstrate this effect on tolerance as measured by rectal temperature. In each experiment, alcohol was previously injected in one room and saline was previously injected in a conspicuously different room. The data from Le et al. (1979) show the second of two experiments reported. The data from Mansfield and Cunningham (1980) show group E at 60 minutes after alcohol or saline injection, the time of peak drug effect. The data from Crowell et al. (1981) show the first of two experiments.

TABLE 1.--Changes in rectal temperature of rats after alcohol injection, comparing the alcohol environment with the nonalcohol environment and including a control group with no prior alcohol exposure

Reference	Environment		No prior alcohol
	Alcohol	Nonalcohol	
Le et al. (1979)	-1.10	-1.83	-2.10
Mansfield and Cunningham (1980)	-0.55	-1.03	-1.13
Crowell et al. (1981)	+0.18	-1.32	-1.36

These three studies agree remarkably well. Although the experimental manipulations controlled only part of the external environment associated with alcohol injections, they were almost completely effective. Tolerance is shown by the smaller decrease in rectal temperature in the alcohol environment than in the control group given no prior alcohol exposure. The compensatory response--counteracting hypothermia in the alcohol environment--is indicated by the much larger decrease in rectal temperature in the nonalcohol environment. All three experiments showed less hypothermia in the nonalcohol environment than in the control group, but this tolerance was much lower than in the alcohol environment.

Table 2 shows a comparison between the alcohol and nonalcohol environments when saline was substituted for alcohol. The increase in rectal temperature in the alcohol environment gives evidence of a conditional compensatory response induced by the alcohol environment in the absence of alcohol.

Other studies indicate that the effects shown in table 1 are quite general. Melchior and Tabakoff (1981) found the same effects; they used mice and measured rectal temperature and duration of loss of the righting reflex caused by alcohol. Tests to show the influence of the same environment do not require the use of exactly the same drug. Cappell et al. (1981) found that after rats developed tolerance to the hypothermic effects of pentobarbital, they showed cross-tolerance to alcohol, but only if the alcohol was administered in the environment that was associated with pentobarbital.

TABLE 2.--Changes in rectal temperature of rats after saline injection, comparing the alcohol environment with the nonalcohol environment

Reference	Environment	
	Alcohol	Nonalcohol
Le et al. (1979)	+0.52	-0.23
Mansfield and Cunningham (1980)	+0.38	+0.09
Crowell et al. (1981)	+0.82	+0.06

BEHAVIORALLY AUGMENTED TOLERANCE

Tolerance has been shown when the animal practices a coordinated motor response in the presence of alcohol. These animals were compared with animals given the same amount of alcohol after the performance sessions. The behavior measured was avoidance of shock on a moving belt (LeBlanc et al. 1975, 1976; Wenger et al. 1980, 1981), rearing on the hind legs during exploration of a Y-shaped maze (Wood 1977), and pressing a lever for food pellets (Chen 1979).

This type of tolerance--i.e., one that is associated with performance--develops gradually. The rate of development depends both on the number and the consistency of occasions when the response is practiced under the influence of alcohol. Table 3 shows data from a study by LeBlanc et al. (1976). When only a few alcohol doses are given, the amount of tolerance increases with increasing consistency of performing under the influence of alcohol. This is not due to more effective tolerance when there is a shorter interval between alcohol doses because after more numerous doses, consistency no longer increases tolerance. After 10 alcohol injections, tolerance was greater in the rats that were given alcohol every second day rather than every day. Tolerance was retarded in the rats that were given alcohol every third day, but it was developing after the fifth drug dose.

A conditional response to the external environment associated with alcohol could explain why the control group, which had previously been given alcohol after the daily sessions, performed more poorly in the test for tolerance. Hinson and Siegel (1980) pointed out that administration of alcohol before the test for tolerance was the customary situation for the group with behaviorally augmented tolerance, but it was a novel situation for the control group.

TABLE 3.--Performance on the moving-belt test reported by LeBlanc et al. (1976). Four groups of rats, given alcohol on different schedules (every day to every fourth day), are compared after different numbers of alcohol injections (3 to 19).

	Percentage change in performance failures			
Alcohol injections	Alcohol every day	Alcohol every second day	Alcohol every third day	Alcohol every fourth day
3d	-18	-10	-04	-01
5th	-27	-17	-11	+03
10th	-43	-62	--	--
19th	-54	--	--	--

Tolerance has been reported to develop, although slowly, in animals that are given alcohol after the performance sessions (LeBlanc et al. 1976). Their occasional tests preceded by alcohol administration may have provided sufficient trials for the animals to learn to associate the conditional compensatory response with the performance situation. The lack of such prior tests may explain why Wenger et al. (1981) found that in the first performance test preceded by alcohol administration, the tolerance caused by alcohol administration for 23 prior days, after the daily performance tests, was slight and not statistically significant.

ACUTE TOLERANCE

A more rapid form of tolerance occurs within the same session. It is shown by recovery of a function at a higher brain-alcohol level while brain alcohol is decreasing in contrast to the loss of the function earlier in the session, when brain alcohol is increasing. A more convincing demonstration is to show that when the alcohol level is kept constant by repeated administrations of small doses or by continuous inhalation, the performance gradually improves.

When a coordinated motor performance is measured, acute tolerance may include a rapid form of behaviorally augmented tolerance. This can account for the large magnitude of acute tolerance found in some studies (reviewed by Littleton 1980). Consistent with this is a finding by Kalant et al. (1978) that acute tolerance was prominent when behaviorally augmented tolerance was highly developed.

Some conditions apparently minimize acute tolerance. Tabakoff and Ritzmann (1979) tested the brain-alcohol levels at the time of loss and regaining of righting reflex of mice after a test dose of alcohol. Acute tolerance was slight and was limited to only some of the strains of mice tested.

TOLERANCE AND WITHDRAWAL

The withdrawal illness appears to constitute persistence of the compensatory tolerance while the drug effect diminishes (Cicero 1980). Withdrawal symptoms were observed in rats that became tolerant to alcohol because they were performing a motor-coordination task while intoxicated (LeBlanc et al. 1975). Withdrawal symptoms were more severe in rats that had previously been subjected to alcohol withdrawal (Baker and Cannon 1979).

Alcohol withdrawal symptoms have been reviewed in laboratory animals (Friedman 1980) and in humans (Turner 1980). Hemmingsen et al. (1979) discuss evidence that the extraordinarily severe illness of delirium tremens, which occurs in some alcoholics, is a symptom of alcohol withdrawal. These reactions indicate that the compensatory response of tolerance may be intense and persistent, in accordance with the prolonged disruptive effects of alcohol. The withdrawal illness can be severe and even lethal, but an adaptive feature is to deter additional alcohol consumption.

Some conditions have differential effects on tolerance and withdrawal. The degree of tolerance to alcohol is limited, reaching a maximum after a few exposures to the drug, and the severity of the withdrawal illness can be increased by larger amounts or longer durations of alcohol exposure. Tabakoff and Ritzmann (1977) found that the alcohol-withdrawal illness was not affected by a drug treatment that counteracted tolerance. In a systematic comparison with pentobarbital, alcohol induced less tolerance but more severe withdrawal symptoms (Boisse and Okamoto 1980). In comparison with alcohol and barbiturates, opioids induce greater tolerance, but the withdrawal illness is less severe (Kalant 1977).

These differential effects on tolerance and withdrawal are attributable to the different situations in which the compensatory response initially occurs in the presence of the drug (tolerance) and persists in the absence of the drug (withdrawal). For example, in comparison with barbiturates and opioids, the intense pharmacological action of alcohol might induce a stronger compensatory response, which is more effectively counteracted by the drug effect during the tolerance phase. This can account for the lesser tolerance but more severe withdrawal illness for alcohol than for other drugs.

EXTERNAL ENVIRONMENT ASSOCIATED WITH WITHDRAWAL

Conditional arousal of alcohol withdrawal is indicated by a study of alcoholics (Ludwig et al. 1974). Craving, working for alcohol, and other reactions were induced by an external environment associated with drinking. A further study showed that the environment associated with drinking had a greater effect on steady drinkers than on binge drinkers (Ludwig et al. 1977). Steady drinkers presumably have more frequent and consistent opportunities to acquire conditional responses associated with the drinking environment.

These studies are based on the assumption that the environment associated with drinking induces a conditional compensatory response, which is manifested as tolerance during alcohol intoxication and later withdrawal. A complication during withdrawal might be the learning of a conditional compensatory response to counteract the withdrawal illness. This might be why the magnitude of tolerance does not always correspond to the intensity of withdrawal. Goldstein (1974) reported that when mice were continuously intoxicated by inhalation of alcohol vapor, the withdrawal symptoms were greatly diminished if there were interruptions in the intoxication. Perhaps the prior withdrawal experiences enabled the mice to acquire a conditional compensatory response to the illness.

A further hypothesis, applicable to humans, is that a conditional compensatory response to the withdrawal illness might cause the withdrawal symptoms to be less severe if withdrawal consistently occurs in a familiar environment that is different from the intoxication environment, such as in the bedroom the next morning. Admission to a hospital might aggravate the withdrawal illness because the conditional compensatory response is weaker in a novel external environment.

CONCLUSIONS

Several experiments on laboratory animals have shown an important role of the external environment in alcohol tolerance. The tolerance was almost completely reversed when the drug was administered in an environment that was not associated with alcohol administration. An opposite response (hyperthermia instead of hypothermia) was induced when saline was administered in the environment associated with previous alcohol administrations.

These findings support the assumption that tolerance involves a compensatory behavioral response that counteracts the direct drug effect. In comparison with external signals received through the sensory receptors, such as visual and auditory signals, the effects of alcohol are intense and long lasting. These attributes of the drug may explain why the conditional compensatory response is induced so strongly by the external environment

before the drug's administration and persists for a long time. A compensatory response that is sufficiently early, intense, and persistent may be necessary to prevent lethal or other damaging effects of the drug.

The use of rectal temperature probably was an important aid in demonstrating the conditional response to the external environment. Body temperature is a continuous, quantitative function that can be measured precisely. This physiological function is sensitive to changes in external and internal environments.

Withdrawal illness is attributable to persistence of the compensatory response, counteracting the alcohol effects, after the drug is no longer present. This is sometimes maladaptive, but it has the adaptive feature of deterring additional alcohol consumption. Exposure of alcoholics to an environment associated with drinking can induce withdrawal symptoms, indicating the occurrence of conditional compensatory responses in the absence of alcohol.

It is logical to expect that the external environment associated with the withdrawal illness might evoke a conditional compensatory response. If alcohol intoxication and withdrawal occur in the same environment, the opposite conditional compensatory responses will counteract each other. Future studies of the conditional responses to withdrawal should associate intoxication and withdrawal with different external environments.

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Relationship Between Physical Dependence on Ethanol and Reinforcing Properties of Ethanol in Animals

Richard A. Meisch

It is commonly assumed that physical dependence on ethanol is an important determinant of ethanol drinking, and it is often thought that subjects continue to drink ethanol partly to avoid withdrawal symptoms. In the past 13 years physical dependence on ethanol has been demonstrated in mice (Freund 1969; Goldstein and Pal 1971), rats (Begleiter 1975; Branchey et al. 1971; Falk et al. 1972), dogs (Essig and Lam 1968), rhesus monkeys (Deneau et al. 1969; Ellis and Pick 1970, Pieper and Skeen 1972) and chimpanzees (Pieper et al. 1972). The results of these and other studies with animals now make it possible to evaluate the role of physical dependence on ethanol.

The results consistently show that physical dependence is not a sufficient condition for maintaining ethanol intake in mice, rats, and rhesus monkeys. For example, when four physically dependent mice were given a choice of drinking a liquid diet containing ethanol or an isocaloric liquid diet containing sucrose in place of ethanol, all four mice selected the solution containing sucrose, and an abstinence syndrome resulted (Freund 1969). Similar results were obtained with rats (Hunter et al. 1974). In one study with rhesus monkeys physical dependence was established by the administration of ethanol through a nasogastric tube. After the monkeys were physically dependent, ethanol administration was stopped, and the monkeys were given access to an ethanol solution. It was thought that they might avoid withdrawal by drinking ethanol, but none did, and withdrawal occurred (Myers et al. 1972).

Physical dependence is also not necessary for high ethanol intake (cf. Mello 1973). In several studies rhesus monkeys were able to self-inject ethanol through an indwelling venous catheter

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(Carney et al. 1976; Karoly et al. 1978; Winger and Woods 1973; Woods et al. 1971). When access to ethanol was limited to 3 hours or 1 hour/day, large amounts of ethanol were reliably self-injected, and physical dependence was not seen. This is not surprising since between sessions the ethanol that was taken during the sessions could be completely metabolized. These findings are not limited to experiments that used the intravenous (IV) route. In other studies where access to ethanol was limited to several hours per day, rats and rhesus monkeys consistently drank volumes of ethanol solutions that substantially exceeded their water intake (Beardsley et al. 1978; Freed et al. 1970; Freed and Lester 1970; Henningfield and Meisch 1978; Meisch and Beardsley 1975; Meisch and Thompson 1973, 1974b,c).

A peculiar characteristic of ethanol is that it is the only drug known that physically dependent humans or rhesus monkeys will stop taking, thereby self-initiating an abstinence syndrome (Deneau et al. 1969; Mello and Mendelson 1972; Nathan et al. 1971; Winger and Woods 1973). In contrast, physically dependent rhesus monkeys will not stop self-administering barbiturates or opioids (Winger and Woods 1973).

With drugs other than ethanol substantial drug-seeking behavior and drug intake can occur in the absence of physical dependence (Spealman and Goldberg 1978; Young et al. 1981). This is true when drugs are available for only short times each day. For example, when access to a pentobarbital solution was limited to 3 hours/day, rhesus monkeys reliably drank concentrations as high as 4 mg/ml (Meisch et al. 1981). When water was substituted for the pentobarbital solution, drinking declined and no signs of withdrawal were observed. Subsequently, when the pentobarbital solution was reintroduced, the monkeys promptly resumed drinking. In this study and many others, drug-reinforced behavior occurred in the absence of physical dependence (Spealman and Goldberg 1978).

Because strong drug-seeking behavior can occur without physical dependence, terms such as psychic or psychological dependence have been introduced (see Goldberg 1976). The definitions of psychic dependence have included words such as "satisfaction" and "pleasure." Such terms have not been helpful because they are hard to define in a scientifically useful way. A different approach to a definition of psychic dependence arises from studies of drug self-administration. In these studies drug-taking behavior is viewed as an instance of operant behavior--i.e., behavior that is controlled by its consequences. Drugs that maintain a response that leads to their administration are classified as positive reinforcers (see Johanson and Schuster 1981). Within this conceptual framework, behavioral dependence can be said to occur when a drug (ethanol included) functions as a positive reinforcer for a particular organism (Goldberg 1976; Meisch 1982; Meisch and Thompson 1974a). Behavioral dependence can be present to varying degrees, and the degree of behavioral dependence can be equated with the strength of the drug as a reinforcer

for a particular individual (Meisch 1982). Under different conditions the same drug can function as either a strong or weak reinforcer. The strength of a drug as a reinforcer probably varies as a function of such factors as the pattern and extent of prior drug use. These statements also apply to ethanol since it is a drug.

It is important to recognize a distinction between behavioral dependence and physical or physiological dependence because in the laboratory the two types can occur independently. Outside the laboratory one is unlikely to find physical dependence in the absence of behavioral dependence. However, behavioral dependence can occur in the absence of physical dependence. Thus, detoxification is not sufficient treatment for excessive and chronic ethanol drinking because it deals with physical dependence and not with behavioral dependence. If behavioral dependence is not changed, the subject is likely to return to excessive ethanol drinking. Because of the failure to distinguish between behavioral and physical dependence, discussions in the literature of the role of dependence have been confusing.

There have been no animal studies of the effects of physical dependence on the effectiveness of ethanol as a reinforcer. Thus, it is not known if ethanol is a better reinforcer when organisms are physically dependent than when they are not physically dependent. This is also true for many other drugs. For example, it is not known whether barbiturates are more effective reinforcers when animals are physically dependent than when they are not. This is an important area in which we lack knowledge and in which future research may prove valuable (for another discussion of some of these topics see Cappell and LeBlanc 1979).

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Methodological Considerations in Studies of Physical Dependence on or Tolerance to Ethanol

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INTRODUCTION

It is well documented that chronic ingestion of ethanol results in physical dependence and functional tolerance as characterized by withdrawal symptoms in animals (Mello 1973) and in humans (Gross et al. 1974). Studies of these actions of ethanol have been sporadically conducted over the past century, but only in the past 2 decades has an attempt been made to rigorously characterize the behavioral, physiological, and biochemical processes associated with tolerance to and dependence on ethanol. Most human studies have generally been limited to characterizations of behavioral and physiological symptoms associated with withdrawal from chronic ethanol ingestion. Recently, however, human studies using ethnic populations (Wolff 1972), families (Goodwin et al. 1974), and twins (Hrubec and Omenn 1981) have provided evidence that alcoholism is influenced by gene action as well as by environmental and social factors. Because of obvious ethical constraints, human studies have limits for determining the underlying mechanisms of ethanol action. Thus, investigators must depend largely on laboratory animals for these studies. Significant progress has been made in understanding the biochemical, physiological, and behavioral consequences of both acute and chronic alcohol ingestion in studies using animals (Kalant 1975). Animal models for studies of alcohol consumption and of the acute actions of ethanol (Erwin and McClearn 1981) have been created using selective breeding techniques (DeFries 1981). These studies include selectively breeding for: (1) differences in central nervous system (CNS) sensitivity to acute doses of ethanol in mice and rats (Rusi et al. 1977; McClearn and Kakihana 1973; Riley et al. 1976); (2) differences in preference for ethanol solutions (Li et al. 1981); and (3) differences in severity of handling-induced seizures after withdrawal from ethanol (Goldstein 1973).

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Some of these selected lines of animals were terminated before they could be used in followup studies, leaving a limited number of selected lines available for use in etiological investigations. To date, animal lines that show differences in rates of acquiring tolerance to, or dependence on, ethanol have not been generated by selective breeding.

Problems encountered in virtually all of the selection studies to date include less than optimal genetic design, resulting in excessive inbreeding and lack of confidence in the results when relationships between the selected trait and other measures are examined (DeFries 1981). Another potential problem encountered in selection studies includes the use of a univariate selection index to define a complex phenomenon.

Ethanol dependence as a pharmacological concept is principally described by measuring withdrawal symptoms. McClearn et al. (1982) have stated that "no one has ever measured dependence; they have been able simply to measure various manifestations of it such as, for example, convulsions upon withdrawal." Over the past decade a number of investigators have devised behavioral methods and physiological indexes for determining chronic alcohol-withdrawal signs. For example, Wood and Laverty (1979) have used withdrawal ratings, which rank the degree of muscle rigidity, irritability, gait, and seizures on a scale of 0 to 5. Similarly, Tabakoff and Ritzmann (1977) used a withdrawal classification with essentially a scale of 0 to 3, indicating no withdrawal, mild, or severe withdrawal, utilizing tremors and spasms and clonic seizures when mice were lifted by the tail, or spontaneous clonic-tonic seizures. Goldstein and Pal (1971) reported a seizure rating on withdrawal that delineated four levels of severity of seizures on handling. Other measures of withdrawal from chronic ethanol ingestion have been proposed and used, and they include a variety of behavioral and physiological assessments (Freund 1969; Majchrowicz 1977). In general, these withdrawal symptoms can be ranked in order of the severity of withdrawal, i.e., "dependence," but the measures do not provide the degree of precision that one desires in studying the biochemical and physiological processes associated with ethanol dependence. Tabakoff and Ritzmann (1977) have shown that changes in body temperature (hypothermia) during withdrawal are correlated with the severity of other withdrawal signs. The time course for hypothermia corresponds well with the time course for heightened seizure susceptibility. Indeed, this withdrawal symptom appears to provide one of the more quantifiable measures of withdrawal.

Since there are many withdrawal symptoms following chronic ethanol administration, determining which withdrawal measure to use in initiating a selective breeding program to provide animal models for studies of ethanol dependence is a major problem. Because of previous problems associated with univariate selection to define complex phenomena, we initiated a study to develop a withdrawal index based on a number of correlated withdrawal signs and to

selectively breed for the severity of ethanol-withdrawal symptoms using this index derived via multivariate procedures.

It is likely that the ethanol-withdrawal syndrome is described by the interaction of the effects of alcohol on various physiological and ultimately biochemical processes. Recently McClearn et al. (in press) have conceptualized the multivariate approach using a Venn diagram, as shown in figure 1. It is assumed that the dashed circle encompasses the pharmacological concept of ethanol dependence--i.e., withdrawal syndrome--and that the closed circles, numerals I through V, represent various specific measures of the alcohol-withdrawal syndrome. As indicated, measures I through V overlap, to varying degrees, with the dashed circle that represents the "dependence" domain. Some of the closed circles overlap each other, but others do not, illustrating that some withdrawal measures may be mechanistically associated but that others may have separate and distinct mechanisms. This illustration is helpful in conceptualizing a multivariate function. However, the real situation is probably more complex and multidimensional where the circles might be viewed as spheres or hyperspheres with multiple possibilities for overlap.

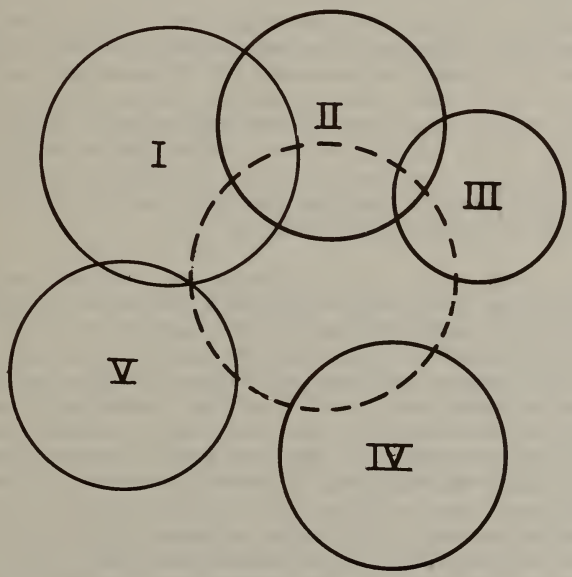

FIGURE 1.--Venn diagram of hypothetical relationships among withdrawal measures and dependence. Modified from McClearn et al. 1982. Reprinted with permission.

Other methodological problems that arise in developing animal models for studying rates of acquisition of alcohol tolerance and dependence are time of treatment, dosage regimen, and method of administration. Various procedures, including inhalation of ethanol vapor (Goldstein and Pal 1971), multiple injections or intubations (Majchrowicz 1975), and liquid diets containing ethanol (Hutchins et al. 1981) have been used to induce dependence. In determining a satisfactory method for the chronic administration of ethanol for selection studies, we chose the oral route because of its greater physiological relevance to "drinking" in humans and to avoid the lung toxicity associated with chronic ethanol inhalation or the peritoneal toxicity associated with multiple intraperitoneal (IP) injections. A liquid diet containing ethanol was chosen instead of repeated intubations because it provided a convenient method of administration and allowed for some self-selection or acceptance of ethanol. The extent of ethanol acceptance might be relevant to acquisition of dependence. For example, Hershon (1977) speculated that continued "drinking" might be provoked by the unpleasantness of withdrawal symptoms and reinforced by the relief afforded by ethanol.

EXPERIMENTAL DESIGNS

In developing the lines of mice that differ in reaction to ethanol withdrawal, we used a multivariate index-selection procedure to encompass as broadly as possible the dependence domain. We used within-litter selection to minimize inbreeding, and replicate high, low, and control lines were established to minimize spurious correlations in the studies of traits that may be related to ethanol dependence. The methods used in developing this selection study have been described (McClearn et al. in press), and only a brief review is presented here.

A number of behavioral and physiological measures were used to characterize the withdrawal syndrome, and several measures that were found to distinguish chronically ethanol-treated mice from controls were used in computing the index. All measures and consumption scores were intercorrelated and subjected to a principal-component analysis. The first principal component loads on a number of measures, each of which is associated with at least one of the domains of ethanol withdrawal and dependence. Several studies were initially done to determine the correlations and covariances that exist among various measures and to examine relationships between the actions of ethanol and ethanol-related behaviors in genetically heterogeneous (HS/Ibg) mice. The foundation population for this study consisted of 200 HS/Ibg mice, with subgroups of mice ranging in age from 75 to 95 days at the beginning of treatment. No response differences were noted within this age range. The animals were housed individually during treatment and testing, and all procedures were done in a specific pathogen-free (SPF) laboratory at the Institute for Behavioral Genetics, University of Colorado, Boulder. Approximately 400 animals were tested in each generation after the S_0.

The animals were maintained for 9 days on a liquid diet containing ethanol as their sole source of food. Water was available ad lib. On days 1 and 2 they received in their liquid diet 10 percent ethanol-derived calories (edc); days 3 and 4, 20 percent edc; and days 5 to 9, 35 percent edc. Food was changed twice daily, and body weight and total ethanol consumption were recorded daily for each animal. The 9-day regimen was chosen because it produces a large variance in withdrawal measures--a necessary feature in a genetic selection program.

After the ethanol diet was removed, water, lab chow, and control liquid diet were available until testing 6 hours later. Ethanol-derived calories were replaced with carbohydrate calories in the control diet. Until the third generation, each animal was given an IP injection of 12.5 percent ethanol at the beginning of withdrawal. The amount injected was based on body weight, amount of 35 percent edc diet consumed during the preceding 6 hours, and previously calculated elimination rate. This injection was designed to give all subjects a blood-ethanol level of approximately 0.3 g/kg when withdrawal began. As a test of this procedure, in addition to the 419 mice tested in the selection program during generation S_3, 155 siblings were concurrently tested without the injection to determine whether equalization of the blood-ethanol levels at the beginning of withdrawal affected our selection index. Results indicated no differences between these groups; therefore, the 12.5 percent ethanol injection, used to equalize blood-ethanol levels at withdrawal, was discontinued after generation S_3.

The following tests were conducted between 6 and 7 hours after the ethanol diet was removed.

Seizure scores	The severity of handling-induced seizures (0 to 3) was measured by the method of Hutchins et al. (1981).
Hole in the wall	A clear plastic (Plexiglas) box, 31.8 × 31.0 × 15.2 cm, is divided into four equal chambers by an X-shaped barrier. Two chambers are lighted, and two are dark in alternating pattern. The chambers are connected by holes in the barrier. Each mouse roamed freely for 3 minutes while the following were measured: number of crossings, partial entries, number of rearings, time of latency until first crossing, cumulative time spent in the dark compartments, seizure severity, urination, and defecation.
Body temperature	Temperature was taken rectally with a probe inserted 2.2 cm at a room temperature of 24.5±1°C.

Vertical screen — This apparatus consists of a 1.22- × 2.44-m wooden frame, covered with hardware cloth (3.2-mm mesh), mounted vertically against a wall, and divided into 5.1-cm squares. Each subject was placed head down on the center of the screen and allowed to roam freely for 3 minutes. The number of squares crossed, number of approaches to each side, cumulative time spent at the top, urination, and defecation were recorded.

The mating design follows the procedure recommended by DeFries (1981) and described by Allen et al. (in press). In this within-litter selection design, the most extreme male and the most extreme female (in the designated direction) from each litter tested within each line are chosen for mating. These animals are then mated randomly within the line. This design decreases inbreeding by as much as 50 percent per generation when compared with mass-selection techniques. From two initial sets (sets 1 and 2) of 10 families each, we have established two severe ethanol-withdrawal lines (SEW_1 and SEW_2), two mild ethanol withdrawal lines (MEW_1 and MEW_2), and two unselected control lines (C_1 and C_2). The selection index was derived by a first-principal-component analysis of the S_0 measures as described elsewhere (McClearn et al. 1982), and preliminary results will be published (Allen et al. in press). The selection index is defined as a sum of weighted standardized scores derived from seven of the measures described: seizure scores, body temperature, hole-in-the-wall crossings, hole-in-the-wall rearings, hole-in-the-wall seizures, vertical-screen crossings, and total amount of ethanol consumed (grams/kilogram body weight). McClearn et al. (1982) have calculated the component loading scores and the coefficients for the seven variables used in the selection index for both male and female mice in the foundation population. From these component loadings and coefficients the weighted scores for the selection index were calculated.

RESULTS AND DISCUSSION

In deriving the selection index, it was of interest to determine whether preference or voluntary ethanol consumption, either before or after chronic ethanol ingestion, was related to withdrawal measures. Using HS/Ibg mice and the 9-day chronic ethanol treatment, Allen et al. (1982a) found no association between preference for ethanol and the acquisition of dependence as characterized by handling-induced siezures; there was no significant correlation between seizures and ethanol preference either before or after ethanol withdrawal. As expected, there was a small but significant correlation between the total amount of ethanol consumed during chronic treatment and the severity of the handling-induced seizures. We concluded that for the design of the selection study the use of preference ratios or the difference between preference ratios before and after withdrawal would not

significantly contribute to the characterization of the dependence phenomenon. Using chronically treated HS mice with appropriate control groups, Allen et al. (1982b) have also shown that the 9-day ethanol treatment did not significantly alter the rate of ethanol elimination in either male or female mice. These results indicate that acquisition of metabolic (dispositional) tolerance is unrelated to the development of dependence, as characterized by withdrawal seizures. Consequently, a measure of dispositional tolerance would not significantly contribute to a selection index for ethanol dependence.

Results summarized in table 1 show the mean values for males and females in the HS/Ibg foundation population for the seven variables used in the selection index. As noted, when ethanol is withdrawn, there are significant sex differences (females are more affected) for each of the variables except hypothermia and vertical-screen crossings (and these share the same trend). Because five of the seven variables showed a significant sex difference, separate principal-component analyses were performed to establish selection indexes appropriate for each sex.

Data summarized in table 2 show the correlations among the variables used in the selection index. These results are of pharmacologic interest in that they clearly demonstrate the relationships among each of the withdrawal measures--e.g., seizures, hypothermia--and the correlation between each withdrawal measure and the total consumption of ethanol over the 9 days of treatment. As expected, the seizure scores increased, and body temperature and the various behavioral measures decreased as the total consumption of ethanol increased. It can be estimated from the correlation coefficients that less than 16 percent of the variance in seizure score or other withdrawal measure is related to the variance in total ethanol consumption; thus, factors other than total ethanol consumption are involved in the ethanol-withdrawal syndrome.

Results from previous experiments (Allen et al. in press) show the response to selection for severe (SEW), mild (MEW), and control (C) withdrawal lines for five generations of mice. The SEW and MEW lines (males and females) are diverging in the mean withdrawal score although the replicate-1 lines (SEW_1 and MEW_1) seem to be diverging less rapidly than the replicate-2 lines (SEW_2 and MEW_2). The C_1 line displays a spurious result, as yet unexplained. However, the C_2 line is responding appropriately, and the disparity between the C_1 and C_2 lines provides an instructive example of the value of replicate lines in a selection study. The realized within-family heritability after five generations of within-family selection has been reported by Allen et al. (in press) to be approximately 0.15. This value is consistent with a narrow-sense heritability estimate of 0.2 to 0.25 and is consistent with heritability values obtained in other selection studies (Erwin and McClearn 1981). The selection response is greater in the SEW lines than in the MEW lines, and several explanations for such asymmetry in selection response are possible. For example, it

TABLE 1.--Male and female foundation population mean (±SEM) values for the seven variables used in the selection index

	Population mean		Significance of sex difference
	Males (N=111)	Females (N=89)	p<
Seizure score	0.645±0.067	0.911±0.085	.01
Body temperature	33.934±0.101	33.859±0.146	N.S.
Hole-in-wall crossings	8.645±0.759	4.611±0.524	.0001
Hole-in-wall rearings	6.891±0.726	3.872±0.501	.001
Hole-in-wall seizure score	0.191±0.058	0.522±0.110	.01
Vertical screen crossings	28.055±1.325	25.600±1.340	N.S.
Total ethanol consumption in grams ethanol/kilogram body weight	97.446±1.452	130.839±3.637	.0001

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TABLE 2.--Correlation matrix of variables used in the composite selection index*

	SEIZ	BODYT	HWX	HWREAR	HWSEIZ	VSX	CON
SEIZ	--	-.37	-.35	-.40	.57	-.28	.38
BODYT		--	.28	.20	-.42	.21	-.22
HWX			--	.40	-.27	.37	-.26
HWREAR				--	-.28	.30	-.27
HWSEIZ					--	-.29	.34
VSX						--	-.14
CON							--

*N=200 for all correlations. All correlations are statistically significant ($p < .01$).

Legend: SEIZ = seizure score; BODYT = rectal body temperature; HSX = hole-in-wall crossings; HWREAR = hole-in-wall rearings; HWSEIZ = hole-in-wall seizure score; VSX = vertical screen crossings; CON = total consumption of ethanol over the 9 days of treatment in grams ethanol/kilogram body weight.

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could be caused by unequal selection differential, directional dominance, selection of heterozygotes, inbreeding depression, or differential allelic frequencies. Any combination of these possibilities could explain why selection pressure has been more successfully applied to the severe ethanol-withdrawal than to the mild ethanol-withdrawal lines.

The data in figure 2 show the mean seizure scores for seven generations of SEW, MEW, and control replicate lines. Similar data for hypothermia are shown in figure 3 and verify that the lines are diverging as expected for the individual withdrawal measures as well as for the withdrawal index. Further evaluation of the individual withdrawal measures from the seventh selected generation shows that selection for mild and severe ethanol withdrawal based on the composite selection index is differentiating mild and severe withdrawal when each variable is evaluated separately.

Based on the number of mating pairs that actually produced the offspring tested in each line during each generation, the inbreeding coefficient was calculated for each line using an estimation procedure based on the effective population size. The values ranged from 6.2 percent to 8.2 percent inbreeding over five generations, which is an average of less than 2 percent inbreeding per generation.

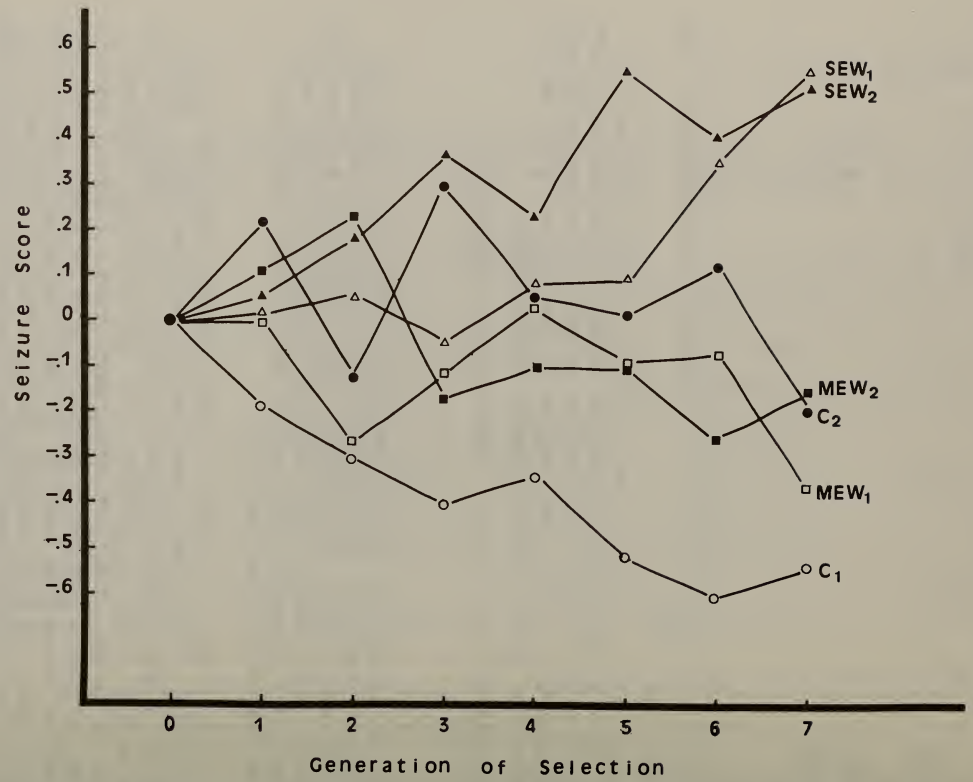

FIGURE 2.--Mean seizure scores for seven generations of SEW, MEW, and control replicate lines (sexes combined)

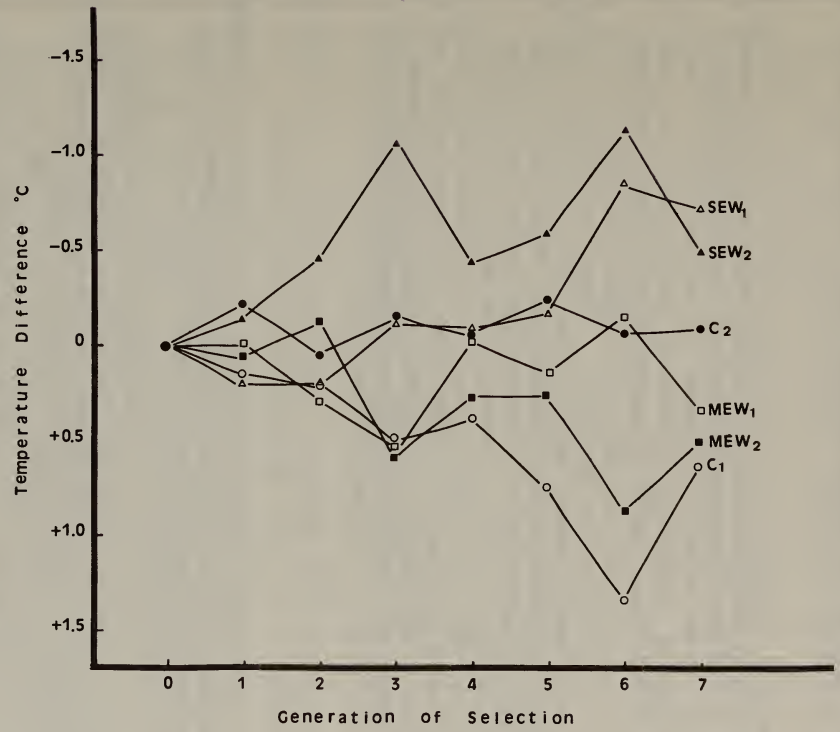

FIGURE 3.--Body temperature loss for seven generations of SEW, MEW, and control replicate lines (sexes combined)

One potential problem with making a selection on the basis of an index derived from first principal-component analysis is the possibility that the component structure may change over various generations--i.e., the weighting coefficients may change over time. In our study, a congruency coefficient was calculated for each generation's uniquely derived component structure as compared with the component structure used in the selection index. These congruency estimates ranged from 0.981 to 0.989 for females and 0.986 to 0.992 for males, indicating that through the S_5 generation no significant change has taken place in the component structure.

Goldstein and Goldstein (1968) and Jaffe and Sharpless (1968) have proposed that tolerance to and dependence on ethanol may be due to common mechanisms involving similar neuronal functions--i.e., these effects of ethanol are mechanistically linked. The studies of Tabakoff and Ritzmann (1977) and Wood and Laverty (1979) have raised questions regarding the possibility of common mechanisms involved in the acquisition of chronic tolerance to and dependence on ethanol. These investigators found that various neurochemical manipulations could dissociate the expressions of tolerance and dependence. Resolution of this controversy is important in the studies of the neurochemical and neurophysiological actions of ethanol. We expect that use of the SEW and MEW (and control) lines of mice should be of value in determining the potential relationships between tolerance and dependence. In addition, these mice should prove valuable as animal models for the study of ethanol dependence and its underlying physiological and chemical mechanisms.

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Evolving Concepts in Ethanol Tolerance and Dependence

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Ethanol tolerance can be defined as an acquired resistance to the effects of alcohol due to prior exposure to this drug. This simple definition does not, however, describe a phenomenon that is simple, and recent studies have indicated that alcohol tolerance may have several distinct components. We have used the terms "environment-dependent" and "environment-independent" tolerance to describe forms of central nervous system (CNS) tolerance that, respectively, do and do not depend for their expression on the circumstances in which the organism receives ethanol. The use of the term environment-dependent tolerance was necessitated by studies that showed that the development of tolerance, or at least the demonstration of the presence of tolerance, could be coupled to the enviromental conditions under which drug administration and testing took place. Both Chen (1968) and LeBlanc et al. (1973) demonstrated that pairing ethanol administration with the performance of a behavioral task increased the rate at which tolerance to the ethanol-induced disruption of the behavior developed. However, the conclusions reached by the two laboratories from their respective studies were not in harmony. Chen (1968) proposed that the tolerance that developed to the impairing effects of ethanol during the chronic treatment consisted of distinct "behaviorally augmented" and "functional" components. LeBlanc et al. (1973) concluded that tolerance produced under various experimental conditions was determined by a singular set of adaptive changes, and only the rate of tolerance development was influenced by the functional demand imposed on the CNS during the period of the drug effect.

Recent studies by Wenger et al. (1981) question LeBlanc's conclusion by suggesting that tolerance cannot be demonstrated to the low-dose effects of ethanol if one does not expose an animal

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to ethanol within the testing situation. The studies of Wenger et al. (1981), Le et al. (1979), Crowell et al. (1981), Mansfield and Cunningham (1980), and Melchior and Tabakoff (1981) have led to the conclusion that Pavlovian conditioning may play an important role in the development of at least one type of ethanol tolerance (i.e., the environment-dependent type). According to this model, each drug administration represents a conditioning trial: the drug administration constitutes the unconditioned stimulus, which, in turn, elicits an unconditioned physiological response(s) from the organism. The total enviromental context in which the drug is given, such as the experimental apparatus, procedures, and injection ritual, serve as conditioning stimuli.

During repeated drug administration, a conditioned response develops in association with those environmental stimuli that have been paired with the unconditioned pharmacological stimulus. This conditioned response may, in certain instances, represent an attempt to compensate for the physiological perturbation produced by the drug. As a result, the conditioned response effectively counteracts the direct effect of the drug, and the observed result of such attenuation of drug action is tolerance. Therefore, as a consequence of repeated drug administration within a consistent predictable environment, tolerance may be demonstrable only in the environmental context with which the drug has been paired. If the drug is administered outside the environment with which it has been associated, the organism will not be tolerant.

On the other hand, environment-independent tolerance may develop in circumstances where conditioning or learning could play but a minimal role. For instance, the administration of ethanol through a liquid diet or by placing animals in a chamber where they inhale ethanol vapor, and later testing them for the effects of ethanol by taking them to a novel environment and injecting them with ethanol, formed a paradigm in which learning would be expected to play only a minimal role in the expression of tolerance. Tolerance did, however, develop under such conditions. We have also demonstrated that even though care was taken to provide equal quantities of ethanol to rats of eight different strains in the inhalation chambers, the rates of tolerance development depend strongly on the strain of the animals that were being tested. Rats of the M-520 strain developed tolerance quite rapidly, while rats of the Fischer 344 strain developed little tolerance throughout the 18 days of exposure to ethanol. Rats of the Maudsley-Reactive strain actually became more sensitive to ethanol's incoordinating effects during the prolonged exposure to ethanol in the inhalation chambers (Culp and Tabakoff 1981).

This comparison of the rates of tolerance development among strains strongly indicates that the development of environment-independent ethanol tolerance is under genetic control. Prior studies that used inbred mice as experimental subjects also demonstrated genetic determinants of certain forms of tolerance (Littleton 1980; Tabakoff and Ritzmann 1979). The studies with mice concentrated on an examination of what may be called acute

[or short-term (Littleton 1980)] tolerance and indicated that C57B1 mice develop tolerance more easily than DBA mice. Some of the studies of short-term tolerance (Littleton 1980) were confounded by repeated testing of the same animal within a consistent environment; thus, in such studies, one cannot distinguish the type of tolerance that was examined. One can conclude that the development of environment-independent tolerance is, like an animal's initial sensitivity to ethanol (Belknap 1980), under genetic control, but little or no information is available on the genetic contribution to environment-dependent tolerance development.

There has been some speculation on the relationships between an animal's initial sensitivity to ethanol and the rate or extent of tolerance development (Tabakoff et al. 1980; Tabakoff and Ritzmann 1979). The data derived from the studies of Crabbe et al. (in press) indicate that a correlation exists between a mouse's initial sensitivity to the hypothermic effects of ethanol and the extent of the development of tolerance to this effect. These data are similar to those from our studies of initial sensitivity and tolerance development in rats; little correlation between initial sensitivity to the incoordinating effect of ethanol and the rate of development of tolerance on this measure was noted, but a significant correlation was found between initial sensitivity and the extent of the acquired tolerance. Whether or not the rate of tolerance development is correlated with other phenomena that accompany chronic ethanol ingestion, such as physical dependence, is considered later in this chapter.

Studies of alcohol tolerance in animals have generally concentrated on the sedative or hypnotic effects of ethanol. Until recently, the low-dose activating effects of ethanol have remained an interesting but little-considered event in terms of tolerance studies. As with the sedative effects of ethanol, significant strain and species differences exist for ethanol's activating effects (Belknap 1980). Ethanol at low doses or during the early rising phase of the blood-ethanol curve increases the locomotor activity in certain strains of mice. DBA and BALB mice are activated by low doses of ethanol, and C57B1 mice show either no effect or behavioral depression as ethanol doses are increased (Belknap 1980; Tabakoff and Kiianmaa in press). Activation of locomotor behavior in rats by ethanol has not been consistently demonstrated (Frye and Breese 1981; Hunt and Overstreet 1977), but this inconsistency may be related to a lack of consideration of genetic variables.

Interestingly, three separate studies (Crabbe et al. 1982; Masur and Boerngen 1980; Tabakoff and Kiianmaa in press) have demonstrated that no tolerance develops to the activating effects of ethanol in mice; in fact, one study (Masur and Boerngen 1980) indicated that the activating effects of ethanol became more pronounced as animals continued to receive daily doses of ethanol. All three studies also demonstrated that the lack of tolerance to the activating effects of ethanol was evidenced under conditions that produced substantial tolerance to the hypnotic effects of ethanol.

The single available study of the development of tolerance to the activating and depressant, or incoordinating, effects of ethanol in rats did show that tolerance developed to both effects of ethanol simultaneously. The incoordinating effects of ethanol were measured in the studies of Hunt and Overstreet (1977) by use of a Rotarod apparatus. However, if tolerance developed to the locomotor-activating effects of ethanol, the diminished activation could, in itself, allow an animal to improve its performance on a rotating drum. This response would appear to represent tolerance to the incoordinating effects of ethanol. Given this possibility, the issue of whether tolerance develops concurrently with the activating and sedating/depressant effects of ethanol in the rat would not be adequately resolved by the experiments of Hunt and Overstreet (1977).

The available data support a preliminary conclusion that tolerance does not develop equivalently to all of the behavior-modifying effects of ethanol. In addition to the studies described above, it has been demonstrated that development of tolerance to the hypnotic and hypothermic effects of ethanol occurs at significantly different rates (Melchior and Tabakoff 1981).

The activating effects of ethanol in animals or humans have, rightly or wrongly, been thought to reflect the reinforcing properties of ethanol (Engel 1977). If this is the case, then the lack of development of tolerance to the activating effects of ethanol in animals is consistent with studies on tolerance development to the reinforcing effects of cocaine and d-methamphetamine (Schuster 1978). In studies that used self-administration of cocaine or amphetamines by monkeys, no tolerance to the reinforcing properties of these two drugs was noted, but significant tolerance developed to the disruptive effects of these drugs on behavior in an operant conditioning paradigm, where animals pressed a bar for a food reward. Although direct and systematic studies of tolerance development to the reinforcing effects of ethanol have yet to be done, studies by Chen (1979), with rats and by Mann and Vogel-Sprott (1981) with humans indicated that tolerance to the disruptive effects of ethanol developed more rapidly or to a greater extent than usual under conditions in which the behavioral disruption interfered with obtaining a reward in an operant-conditioning paradigm. Thus, ethanol may fall into a general category of drugs to which tolerance develops in accordance with the reinforcement-loss hypothesis proposed by Schuster (1978). This hypothesis states that: "Tolerance will develop in those aspects of the organism's behavior repertoire where the action of the drug is such that it disrupts the organism's behavior in meeting the environmental requirement for reinforcements. Conversely, where the actions of the drug enhance, or do not affect, the organism's behavior in meeting reinforcement requirements, we do not expect the development of tolerance." Based on this hypothesis, one would expect little or no tolerance to the development of the reinforcing properties of ethanol per se.

In another context, one can speculate on tolerance development in systems that are much less complex than those required for the types of behavior that Schuster was examining. If one considers the interaction of ethanol with a single neuron or a neuronal network, the "behaviors" that may be monitored are the electrophysiologic events that relate to the transmission and conduction of information. Traynor et al. (1977) have demonstrated that tolerance develops to the disruptive effects of ethanol on stimulus-elicited post-tetanic potentiation, which was produced in an identified synapse of the marine invertebrate Aplysia californica. However, if ethanol was added to the preparation under conditions where no stimulation was being applied to the terminal region, no tolerance developed. Therefore, if no functional requirements were being placed on the system in the presence of ethanol (i.e., no stimulation was being applied), the system did not become tolerant.

The need for proper neuronal stimulation in the presence of ethanol to produce tolerance also applies to the mammalian CNS. Our early studies on the effects of the neurotoxin, 6-hydroxydopamine (6-OHDA), on tolerance development demonstrated that partial destruction of noradrenergic (NE) systems in brains of mice prevented the development of ethanol tolerance (Tabakoff and Ritzmann 1977). In these studies, the 6-OHDA-treated and control animals had quite similar blood-ethanol levels throughout the chronic period of ethanol administration, but the 6-OHDA-treated mice did not develop tolerance to the hypnotic effects of ethanol, whereas the control mice became quite tolerant to the test dose of ethanol. Our recent studies and those of Le and coworkers (1981) clearly show that the NE systems may not be uniquely important for maintaining proper neuronal activity in the CNS for tolerance development. The activity of at least serotonergic (5=HT) as well as NE neurons while ethanol is present in the brain is necessary to "prime" the development of tolerance.

There are significant species differences regarding the neuronal systems that are important for priming tolerance, and there are significant differences within species as to how environment-dependent and environment-independent forms of tolerance are affected by various neurotoxins. The studies of Le et al. (1981) and Khanna et al. (1979) demonstrated that destruction of 5-HT neurons in brains of rats will slow the development of tolerance to the incoordinating and hypothermic effects of ethanol in this animal. Destruction of NE neurons alone has little effect on tolerance development, but if it is coupled with the destruction of 5-HT neurons, tolerance development is completely blocked in the ethanol-treated rats. The tolerance assessed by these workers was reported to be "physiological," or in our terminology, environment-independent tolerance.

In mice, either the selective destruction of 5-HT or NE neurons blocks or postpones the development of environment-independent tolerance. On the other hand, destruction of 5-HT neurons

accelerated the development of environment-dependent tolerance in mice, and destruction of NE neurons slowed the development of the environment-dependent tolerance (Crabbe et al. 1982). These studies provide an initial indication that the various forms of tolerance are under differential neuronal control.

Some of the emerging characteristics of CNS alcohol tolerance are:

(1) Alcohol tolerance is a multifaceted phenomenon, consisting of components that can be influenced or controlled by Pavlovian conditioning and components that can develop independently of behavioral or environmental manipulations.

(2) Tolerance development seems to be influenced by individual genetic constitution. This is particularly true of environment-independent tolerance. There is also some evidence that the genetically determined initial sensitivity of an animal to alcohol predicts the extent of development of certain forms of tolerance.

(3) Tolerance to the various effects of ethanol develops at different rates or may not develop at all. An intriguing possibility is that tolerance to the reinforcing effects of ethanol may not develop under the usual conditions of ethanol administration.

(4) Tolerance may develop only in systems that are carrying out a function that is affected by ethanol. If ethanol is in the system when the affected function is not being initiated or proper stimulus is not being applied, no tolerance will develop in that function. In other words, the presence of ethanol is a necessary but not sufficient event to produce CNS tolerance.

The above conclusion regarding the genetic determinants of tolerance indicates that biochemical processes are involved in the development of resistance to ethanol's actions. The membrane hypothesis of ethanol's action, which states that the acute inebriating effects of ethanol are a reult of the perturbation of the order of the lipids of the neuronal membrane, has also been, until now, the only reasonable biochemical explanation for the development of tolerance (Chin and Goldstein 1977). Neuronal membranes, taken from brains of mice that had been chronically fed ethanol, demonstrated a resistance to the perturbation of membrane lipids, which was witnessed in neuronal membranes prepared from brains of control (ethanol-naive) mice (Chin and Goldstein 1977). This physicochemical demonstration of tolerance must, however, be substantially expanded before it can be accepted as the basic explanation of any or all forms of alcohol tolerance. The chemical change has to be correlated in time and extent with demonstrations of tolerance to the various physiologic and behavioral effects of ethanol, and some explanation must be advanced as to how this singular chemical event can be responsible for the multifaceted nature of alcohol tolerance.

The seeming need for a parsimonious explanation of all of the chronic effects of ethanol has not only maintained the search for a singular biochemical determinant of all forms of tolerance but has also resulted in a general acceptance of the hypothesis that the biochemical determinants of tolerance are also the determinants of physical dependence on alcohol. Several recent studies have brought the biochemical equivalence of tolerance and physical dependence into question. The destruction of brain NE neurons in mice has been shown to block the development of tolerance, but physical dependence, as measured by tremor and convulsions after ethanol withdrawal, was unaffected by the manipulation of the NE systems (Tabakoff and Ritzmann 1977). In studies that attempted to measure the development of tolerance and physical dependence, no evidence could be generated on the equivalence of the time course of development of tolerance and dependence (Majchrowicz and Hunt 1976), and no evidence has been generated to indicate that aspects of ethanol physical dependence can come under the control of environmental contingencies, as can certain forms of ethanol tolerance. On the other hand, symptoms of physical dependence on morphine have been shown to be sensitive to manipulation by classical conditioning techniques (Siegel 1979).

There is, however, one clear electrophysiologic demonstration of the classic relationship that was proposed between tolerance and physical dependence (Jaffe and Sharpless 1968). Ethanol has been shown to suppress rapid-eye movement (REM) sleep if administered in quantities sufficient to cause sedation or hypnosis (Mendelson 1979). After several days of ethanol ingestion by rats, REM sleep returned to relatively normal levels (i.e., tolerance developed), but if ethanol administration was terminated, a significant increase in the quantity of REM sleep (REM rebound) was noted in the animals that were withdrawn (Mendelson 1979). Beyond this phenomenon, the available evidence leaves open the question of whether biochemical or physiologic changes responsible for ethanol tolerance are in any way determining signs of physical dependence on ethanol.

Although the study of ethanol tolerance and physical dependence is intrinsically interesting to those who are curious about the repertoire of adaptive responses inherent in mammalian physiology, are tolerance and physical dependence significant within the context of human alcoholism? Alcoholism is defined by the presence of tolerance and physical dependence, but some researchers (Mendelson and Mello 1979) have doubted the significance of these phenomena as determinants of aberrant drinking behavior. Studies on the relationship between physical dependence and ethanol intake have demonstrated that ethanol may not be consumed by humans or animals to prevent symptoms of withdrawal (Mello and Mendelson 1977). On the other hand, little work has been devoted to generating information on the way that alcohol tolerance may affect a person's or an animal's drinking behavior. If tolerance to the depressant and reinforcing effects of ethanol develops in a differential manner, an individual might consume more ethanol under conditions where tolerance to the depressant

effects of ethanol allows for greater ethanol intake. If, however, tolerance does develop to the reinforcing effects of ethanol, greater intake might result in an attempt to generate past levels of reinforcement. On the other hand, development of tolerance to the reinforcing effects of ethanol might also result in the extinction of behavior that leads to ethanol intake. In any case, there are genetically determined (i.e., biochemical) individual differences in a person's ability to develop tolerance. If tolerance predisposes an individual to an increase in ethanol intake, the understanding and control of the biochemical variables may lead to the control of pathological drinking practices.

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ENDOCRINE EFFECTS OF ETHANOL

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Overview

David H. Van Thiel

During the past 2 decades much new information on the effects of ethanol use and/or abuse on endocrine functioning has been generated. Most importantly, most of this new information was developed as a result of prospective studies of the acute and chronic consequences of ethanol exposure on individual endocrine systems. Three areas of interest have been: the pituitary-adrenal axis, the hypothalamic-pituitary-gonadal axis, and the gastrointestinal tract and its many hormones.

Because of this focus, the present section on endocrine function and alcohol effects also focuses on these three areas. Specifically, the effects of ethanol on the pituitary-adrenal axis are addressed by Crabbe and his colleagues (pituitary effects), Fabre (mineralocorticoid effects), and Van Thiel and coworkers (glucocorticoid effects). The effects of ethanol on hypothalamic-pituitary-gonadal function and conversely the effects of such endocrine functioning on ethanol pharmacodynamics and spontaneous drinking rates in women are addressed by Zeiner (female sex steroids and alcohol pharmacodynamics), Gavaler (an epidemiologic examination of the adverse effects of ethanol on gonadal and sex steroid-responsive tissues), and Mendelson and colleagues (alcohol effects on female reproductive hormones). Finally, ethanol and its effect on gut hormones is discussed by Shanbour.

THE PITUITARY-ADRENAL AXIS

Crabbe and his associates report important genotypic differences in the pituitary content of ACTH, β-endorphin, and α-MSH (melanophore stimulating hormone) in several inbred strains of mice. Coupled with this important observation and relevant to the continuation of such studies in animal models of alcohol exposure, they also report genotypic variation in the severity of physical dependence to alcohol and the consequences of alcohol withdrawal

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in such animals. Whether such findings also extend to humans and explain, at least in part, the differences in response to ethanol and alcohol withdrawal among different population groups remains an intriguing possibility. Should such be the case, unique and specific methods or approaches for modulating ethanol use and possibly ethanol abuse and ethanol-withdrawal responses in humans might be forthcoming. Clearly, this research area will continue to be of considerable interest to alcohol experimentalists and clinicians who care for alcoholic patients.

The biphasic effects of alcohol on aldosterone secretion are discussed in detail by Fabre. In both animal and human studies, he has been able to demonstrate an enhancement of mineralocorticoid secretion by low-dose ethanol (50 to 200 mg/dl) and an inhibition of such secretion with high-dose ($\geq$300 mg/dl) exposure. Most importantly, however, particularly for the interpretation of human studies, is his finding that continued alcohol ingestion over days, at levels easily achievable by humans, is not followed by continued mineralocorticoid hypersecretion (aldosterone) by the zona glomerulosa. As a result, no alcohol-induced syndrome of mineralocorticoid excess has been identified or is expected.

In contrast to the failure to identify or even suspect that a syndrome of alcohol-induced mineralocorticoid excess exists is the finding by several groups, particularly those in Europe, that a syndrome of alcohol-induced glucocorticoid excess does indeed exist. Relevant to this latter finding, Van Thiel presents a historical review of the findings of various investigators who examined the effects of ethanol on the pituitary-adrenocortical (glucocorticoid) axis, using both humans and animals as their experimental model. The evidence for a direct stimulatory effect of ethanol on the central components (hypothalamus and pituitary) of this endocrine axis as well as the alternative, albeit not necessarily conflicting data, that ethanol and more probably acetaldehyde can act as a direct adrenal glocucorticoid stimulant is presented. Finally, a scheme for differentiating the alcoholic with pseudo-Cushing's syndrome from depressed patients and chronically stressed patients with nonalcoholic pseudo-Cushing's syndrome is presented. The scheme is based on empirical observations concerning differences in the subjects' responses to various pharmacologically provocative agents. This scheme should be particularly valuable to clinicians who are faced with the difficult problem of distinguishing true Cushing's syndrome from the various pseudo-Cushing's syndromes that might occur in alcohol-abusing patients.

THE HYPOTHALAMIC-PITUITARY-GONADAL AXIS

A thorough discussion of the reported differences in alcohol pharmacodynamics observed between males and females is presented by Zeiner. Moreover, the data available concerning the effects of steroidal and nonsteroidal estrogens, oral contraceptives, and

pregnancy on spontaneous ethanol consumption rates observed in female animals and humans are presented. Finally, in light of the preceding empirical findings, the recently noted effects of androgens and estrogens on hepatic alcohol dehydrogenase (ADH) activity are discussed. Gavaler extends this discussion by applying epidemiologic methods to animal studies, examining the effects of ethanol feeding on gonadal function and mass and sex-steroid responsive tissues. She reports that male animals ingest about twice the alcohol that a female animal does, but when intake is corrected for body mass, the intakes are equal for the two sexes. More importantly, she points out that testicular atrophy is extremely common (>90 percent prevalence) in animal models of alcohol ingestion and does not increase further as a result of additional ethanol consumption. In contrast, ovarian atrophy occurs with increasing frequency as alcohol intake increases. As might be expected, at least initially, sex steroid tissue atrophy, particularly in females, also occurs with increasing frequency as the ethanol intake increases.

Mendelson and associates present their new and exciting data concerning the effects of alcohol administration on the pituitary and ovarian hormones of the female. Most exciting are their observations on female monkeys, who have chronically self-administered ethanol and as a result become anovulatory. The single animal that died had markedly "atrophic" ovaries (absent corpora lutea but normal follicles), uterine atrophy, lack of peripheral estrogenization, and a pituitary containing basophilic cells that may represent Crooke's cells. This last finding has been seen in the pituitaries of humans who have died of chronic alcohol abuse.

EFFECTS OF ETHANOL ON GUT HORMONES

Shanbour reports her extensive and interesting studies on the endocrine effects of ethanol, examining the effects of ethanol exposure on gastric acid and gastrin secretion. She also presents an overview of the data available on the responses to acute and chronic ethanol administration of gut hormones such as CCK (cholecystokinin), secretin, insulin, and glucagon.

This section on endocrine effects of ethanol abuse appears to represent the state of the art in the area by those who have been most responsible for it. By and large the data are new and should provide a good starting point for academic or clinical research.

Effects of Female Sex Steroids on Ethanol Pharmacokinetics

Arthur R. Zeiner

Several kinds of evidence suggest that sex steroids may be involved in modulating ad lib alcohol consumption, ethanol pharmacokinetics, and acetaldehyde pharmacokinetics. Of increasing concern and interest are both the effects of ethanol on endocrine function and, conversely, the effects of endocrines on ethanol pharmacokinetics. In the past decade, knowledge of the effects of ethanol on endocrine function has greatly increased (Badr and Bartke 1974; Cicero 1980a; Cicero and Badger 1977a,b; Cicero et al. 1979; Ellingboe and Varanelli 1979; Gordon et al. 1976; Mendelson and Mello 1974; Mendelson et al. 1977, 1978; Rubin et al. 1976; Van Thiel et al. 1973, 1974a, 1975; Ylikahri et al. 1974). Such effects have been worked out mainly on male populations since the effects are most dramatic and noticeable in males (Cicero 1980a). In males, chronic alcohol use leads to testicular atrophy, impotence, gynecomastia, changes of body hair, and feminization (Rather 1947; Van Thiel et al. 1974b; Van Thiel and Lester 1976). Decreases in testosterone are demonstrable even after a single dose of ethanol (Cobb et al. 1980, 1981). Excellent reviews exist documenting these changes in male endocrine function related to ethanol ingestion (Kakihana and Butte 1979; Lester et al. 1979; Merry 1976; Van Thiel 1980, 1982; Van Thiel et al. 1981; Wright 1978; Ylikahri et al. 1980).

Conversely, whereas a sizable data base exists on alcohol-endocrine interactions in males, such data are sparse and largely unavailable for females. Further, data on the second question--the effects of endocrines on ethanol pharmacokinetics--were, until very recently, nonexistent.

Research on alcohol tolerance and dependence has taken a new turn with the discovery that the endocrine system might be involved in the development and modulation of tolerance (Cicero 1982). A series of papers from four independent laboratories focused on the question at approximately the same time. In sum, the experiments demonstrate that testosterone inhibits alcohol metabolism, probably via action on the alcohol dehydrogenase (ADH) system (Messiha and Lox 1981; Messiha et al. 1980c; Cicero et al. 1982; Mezey et al. 1980; Mezey and Potter 1982; Rachamin et al. 1980). Mezey and Potter (1982) further elaborated on the mechanisms involved and demonstrated an effect of androgenic steroids on liver ADH activity and ethanol metabolism.

Both data and interest in the effects of endocrines on ethanol pharmacokinetics are more recent and less well developed. This is so because until very recently almost all of the research as well as treatment programs for alcoholism were worked out on males on the assumption that such findings would be directly applicable to females. Recent publications have questioned this (NIAAA 1980; Kalant 1980). Data on the literature indicate that female alcoholics drink fewer drinks and smaller amounts of alcohol than do male alcoholics (Wechsler 1980; Fabian et al. 1981; Parsons and Farr 1981). A series of studies suggests that female alcoholics have a greater risk for developing liver disease, after lower alcohol consumption and a shorter duration of drinking, than do male alcoholics (Galambos 1972; Gavaler 1982; Krasner et al. 1977; Lelback 1974; Pequignot et al. 1974; Wilkinson et al. 1969). In a review, Galambos (1972) suggests a role for estrogen in the increased mortality of female alcoholics from liver injury. The information that was developed, although fragmented and incomplete, has implications for both treatment and research as well as for the development of tolerance and dependence.

Might there be a biological basis, in part, for gender differences in the incidence of alcoholism, cirrhosis of the liver, and, perhaps, the development of tolerance and dependence? This paper focuses on: gender differences in alcohol and acetaldehyde pharmacokinetics and variation in ethanol pharmacokinetics as a function of variation in female sex steroids.

Various experimental methods are available to researchers, and they vary in the rigor and potency of the inferences that can be made. The most direct experiments are those performed with animal models, which permit experimental variation of hormones and determination of concomitant changes in alcohol pharmacokinetics. Human studies are largely restricted to hormonal variation via: (1) oral contraceptives, (2) pregnancy, (3) day of menstrual cycle, (4) menopause, and (5) removal of ovaries without hormone supplements for medical reasons.

ANIMAL STUDIES

Aschkenasy-Lelu (1960a,b), in two studies, demonstrated that during estrus, at a time of elevated estrogen concentration, rats will consume less alcohol than at other times. In a clean study, Tumbleson et al. (1981) studied voluntary alcohol consumption in six 2-year-old Sinclair miniature sows over 17 estrus cycles (1 year). The animals showed a clean sinusoidal variation in voluntary alcohol intake. The amplitude of the variation was more than fivefold, with the low point occurring during estrus. Although not conclusive, these data strongly suggest that female sex hormones may be involved in voluntary alcohol consumption.

Another way to manipulate female sex steroids indirectly is via pregnancy. Thus, is there a reliable decrease in ethanol consumption during pregnancy? Several studies bear on this point.

Randall et al. (1980) demonstrated that C57BL mice decreased their ethanol intake during pregnancy and while lactating. Elton and Wilson (1977) extended the generality of these findings by showing that three out of four pregnant pig-tailed macaques also decreased their ethanol consumption after conception. Sandberg et al. (1982) performed three replications of an experiment with pregnant Wistar rats to further delineate the relationship between pregnancy and alcohol consumption. They demonstrated significant decreases in ad lib alcohol consumption in pregnant rats. Nonpregnant rats did not alter their ad lib alcohol consumption significantly over the same time. The decrease in ethanol consumption among pregnant rats was most profound in the 4 days before giving birth. Data from these experiments in different species were also consistent in indicating that ad lib alcohol consumption decreases during pregnancy when female sex steroids are increased.

In two recent experiments, Forger and Morin (1982) and Morin and Forger (1982) somewhat cloud and complicate simple interpretations of alcohol preference in rodents related to hormonal fluctuations via ovariectomy, estrus cycle, and/or pregnancy. Contrary to preference data from human studies and some animal studies, they show that intact animals with higher levels of female sex steroids prefer and consume more alcohol than do ovariectomized animals (Forger and Morin 1982). Their data are, however, consistent on finding decreases in ethanol preference and consumption in pregnant and lactating rats. In their second paper (Morin and Forger 1982) they implicate the adrenals and corticosterone in some of the observed effects.

Direct manipulation of hormones via castration and/or supplemental injection is a powerful test of the hypothesis that hormones modulate ad lib ethanol consumption. Early tests of the hypothesis were done by Mardones (1960), who noted that diethylstilbesterol (DES) significantly reduced ethanol intake in both normal and gonadectomized males and females. However, he could not demonstrate such effects with testosterone. Along these lines, Emerson et al. (1952) found that high doses of estradiol benzoate reduced ethanol consumption in deer mice. Progesterone, even in massive doses, did not alter ethanol preference in the same species. Ericksson (1969) used 3-month-old female rats to test for voluntary alcohol consumption as a function of ovariectomy and contraceptive hormone supplements. Prior to ovariectomy, the animals did not differ from controls in ad lib alcohol consumption. Ovariectomy led to a small decrease in ethanol consumption. Contraceptive hormones strongly inhibited ad lib ethanol consumption of both ovariectomized and control animals.

Rachamin et al. (1980) tried to elucidate more directly the mechanism of these observed effects. In a series of experiments, they tested both male and female spontaneously hypertensive rats (SHR). They established that young (4-week-old) male and female SHR rats did not differ in ethanol metabolic rate or hepatic ADH activity. Then they showed that: (1) the rate of ethanol

metabolism decreases in male SHR rats but not in female SHR rats at a time coinciding with onset of puberty (between 4 and 10 weeks of age); (2) ADH activity also decreases with onset of puberty in males; (3) castration of male SHR rats prevents both the decrease in ethanol metabolism rate and the decrease of ADH activity related to onset of puberty, but ovariectomy had neither of these effects in female rats; (4) chronic administration of testosterone to castrated male and female SHR rats decreases both the rate of ethanol metabolism and ADH activity to values similar to those found in mature males; (5) chronic administration of estradiol-17 to male SHR rats results in increases in ethanol metabolism and ADH similar to those found in female SHR rats; and (6) in the intoxicated, chronically ethanol-fed male SHR rats, serum testosterone concentrations are significantly depressed. In vitro testosterone has no effect on hepatic ADH activity of young male and female SHR rats. The authors conclude that in the male SHR rats, ethanol metabolic rate appears to be limited by ADH activity. This, in turn, is modulated by testosterone. Testosterone has an inhibitory effect and estradiol has a testosterone-dependent stimulatory effect on ADH activity and ethanol metabolic rate in these animals.

Messiha (1981) also studied possible mechanisms of action of female sex steroids on voluntary ethanol consumption of adult male and female Sprague-Dawley rats. Animals received either 0.3 mg/kg estradiol by intraperitoneal (IP) administration or ethinyl estradiol 0.3 mg/kg via IP. At 24 hours after drug administration the animals were sacrificed, and their liver ADH and ALDH (aldehyde dehydrogenase) values were determined enzymatically. Acute administration of both estradiol and ethinyl estradiol resulted in decreased voluntary ethanol intake in both male and female rats. The enzymatic assay demonstrated an inhibitory effect of sex steroids on L-M-ALDH (liver mitochrondrial aldehyde dehydrogenase). The findings are consistent with the hypothesis that there exists a hepatic-gonadal link related to alcohol preference and that the probable mechanism of inhibitory action of estrogenic compounds is related to inhibition of hepatic L-M-ALDH. In three other studies, Messiha and collaborators followed up on these findings (Messiha 1980; Messiha et al. 1980a,b). Results of these studies are consistent with the view that the mechanism of action involves inhibition of ALDH by female sex steroids, a buildup of acetaldehyde after an acute alcohol dose, and a disulfiram-like aversive reaction.

SUMMARY OF ANIMAL STUDIES

Even though a wide variety of experimental manipulations were used in the animal studies reviewed to accomplish hormonal variation (measurement over estrus cycles, pregnancy, castration, supplemental injections, and hormonal implants), the results were remarkably consistent. In sum, the animal studies indicate that increases of female sex steroids, accomplished by whatever means, result in decreased ad lib ethanol intake by various animal

species. In some of the experiments, concomitant tests for hepatic ADH and ALDH function were also done. Data from these studies suggest that the mechanism of action of decreased ad lib ethanol intake with increases in female sex steroids may involve decreases or interference with alcohol- and/or acetaldehyde-metabolizing activities. The data allow for other interpretations as well, but at present the most parsimonious interpretation that fits all of the data concerns interference with alcohol and acetaldehyde metabolism by female sex steroids via inhibition of ADH and ALDH activity.

HUMAN STUDIES

Human studies on modulating ad lib ethanol intake and ethanol pharmacokinetics by female sex steriods are not and cannot be as direct as animal studies. However, approximations can be made. The studies to be summarized have tested pregnant and nonpregnant females, postmenopausal patients, patients with hystero-oophorectomy, patients on oral contraceptives, and patients at different times during their menstrual cycles.

EPIDEMIOLOGICAL DATA

Little et al. (1976), in an epidemiological study, noted a sharp decrease in ethanol consumption among pregnant females. The effect did not seem to be due to education about fetal alcohol syndrome (FAS) or fears about damaging the developing fetus. Rather, patients cited adverse physiological effects as the major reason. In a followup study, Little and Streissguth (1978) demonstrated that alcoholic women will also decrease their voluntary ethanol intake when they become pregnant. Sokol et al. (1981) also found that pregnant females will decrease their ethanol consumption. However, this is by no means true for all pregnant females. On the basis of the Michigan Alcohol Screening Test (MAST), pregnant females could be segregated into two groups. Positive responders significantly decreased their alcohol intake as pregnancy progressed, but negative responders did not.

Another approach to the relationship between female sex steroids and ethanol pharmacokinetics questions whether there are difference in ad lib alcohol consumption among females who are taking oral contraceptives versus females who are not. Jones et al. (1976) found that women who are taking oral contraceptives drink significantly less alcohol than do females who are not taking them. Zeiner and Farris (1979) extended the generality of these findings by studying Native American females who were taking oral contraceptive steroids and a group that was not on oral contraceptive steroids. The groups did not differ in age, education, socioeconomic level, height, or weight. However, the group that was taking oral contraceptive steroids demonstrated significantly lower ad lib alcohol consumption than did the controls. These human studies suggest that altered hormone levels induced by

either pregnancy or oral contraceptive steroids reduce or eliminate the desire to drink alcohol or make it aversive to do so.

Medical histories indicate that female alcoholics have a significantly higher incidence of obstetric and gynecological disorders than do nonalcoholic controls (Jones-Saumty et al. 1981; Jung and Russfield 1972; Lloyd and Williams 1948; Pincus et al. 1951; Ratnoff and Patek 1942; Wilsnack 1973). Although such data do not allow inferences about cause and effect (whether alcoholism causes obstetric and gynecological problems or whether obstetric and gynecological problems predispose to alcoholism), the results are consistent with what has been found in males and suggest that predominantly obstetric and gynecological problems are due to excessive alcohol consumption.

GENDER DIFFERENCES AND MENSTRUAL CYCLE ETHANOL INTERACTIONS

Jones and Jones (1976a,b) tested 20 women twice at different times during their menstrual cycles (days 1, 14, or 27). They also tested 10 males twice at 2-week intervals. They found that women reached a significantly higher peak blood-alcohol concentration (BAC) than did men. Further, the highest peak was attained by women during the premenstrual period. In these experiments, two males and two females were also compared on peak BAC at three different ethanol doses. Results replicated the single-dose study, with females reaching higher peak BACs at all doses than males.

Marshall et al. (1982) tested 9 women and 10 men with 0.5 g/kg ethanol. Time to reach peak BAC, peak ethanol concentration attained, elimination rate (milligram/hour/kilogram), and volume distribution were determined. Groups did not differ in time to peak BAC, but they differed reliably on both peak BAC and volume of distribution. Females reached a higher peak BAC than did males. When the mean concentration of ethanol per liter of total body water was adjusted in males mathematically to that observed in females, the differences in peak BAC between genders disappeared. The authors conclude that the differences in ethanol pharmacokinetics observed between genders can be explained by differences in body composition.

Sutker (1982) presented preliminary data also suggesting that females reach a higher peak BAC than do males for equivalent ethanol doses. Zeiner et al. (1983) tested 79 adult social drinkers (38 females and 41 males) equated on age, drinking history, and lean body mass. Although there was a trend for higher peak BACs to an acute ethanol dose (0.52 g/kg ethanol) in females than in males, the results were not reliably different. The authors did find higher peak breath acetaldehyde concentrations in males than in females. Perhaps the failure to find the effect in the study with the largest N was due to the control over lean body mass (groups were identical). This, in large

part, canceled gender differences in body composition. Kegg and Zeiner (1980) tested for oral contraceptive effects on ethanol pharmacokinetics, acetaldehyde, and cardiovascular measures in two group of seven Caucasian females who either were or were not taking oral contraceptives. In counterbalanced order, subjects were tested twice (on day 1 and day 26 of the menstrual cycle). The ethanol dose was 0.52 g/kg administered in a 1:4 orange drink after a 4-hour fast. Alcohol and acetaldehyde concentrations were determined from breath samples via gas chromatography. Subjects reached a reliably higher peak BAC on day 1 (the first day of menstrual flow) than on day 26. Acetaldehyde concentration at peak BAC was significantly higher on day 26 than on day 1. Acetaldehyde concentration also increased reliably more from day 1 to day 26 in the oral contraceptive group. The oral contraceptive group also had reliably higher diastolic blood pressures than the control group.

In a followup experiment, Zeiner and Kegg (1981) investigated the relationship between oral contraceptives, day of the menstrual cycle, and ethanol pharmacokinetics in 20 Caucasian females. Groups did not differ reliably from each other in age, height, weight, lean body mass, education, or alcohol consumption. Methodology, procedures, and doses were the same as in the previous study. The control group reached a reliably higher peak BAC than did the group that was taking oral contraceptives. For both groups, peak BAC values were significantly higher on day 1, when female sex hormone levels are low, than on day 24, when both progesterone and estrogen concentrations are elevated.

DISCUSSION

Data from a growing number of experiments suggest that sex steroids affect ethanol pharmacokinetics, that there may be gender differences in peak BAC levels for an equivalent acute ethanol dose, and that prolonged alcohol abuse leads to obstetric and gynecological problems. Thus, the results are consistent with female sex steroids in establishing a relationship with ethanol pharmacokinetics. The clearest results are seen with estrogen. There are not enough data to draw clear inferences with progesterone. Very recent data with testosterone indicate that it inhibits liver ADH and ethanol metabolism.

Both human and animal data obtained by manipulating female sex steroids in various ways are consistent in indicating that increased estrogen levels inhibit ad lib alcohol consumption. Some data suggest that the basis for this effect may involve the development of an aversive reaction, akin to that seen with disulfiram, via inhibition of hepatic alcohol dehydrogenase and/or acetaldehyde dehydrogenase functioning. Available human and animal data are both consistent with this hypothesis, although more data are needed for firmer conclusions.

The observations that females develop liver problems earlier, after fewer years of drinking, and after drinking smaller amounts of alcohol than males, may also be related to estrogen.

It is interesting that the basis for an aversive form of behavioral treatment for alcoholism, the disulfram-alcohol reaction, involves the buildup of acetaldehyde and concomitant aversive autonomic reactions. There are indications that racial differences in biological sensitivity may also involve a relative inability to metabolize acetaldehyde (Zeiner 1981). Perhaps gender differences in rates of alcoholism and ad lib alcohol consumption may have a similar basis. If this is true, then large segments of data related to alcohol consumption would have a parsimonious explanation.

SUMMARY

Both human and animal data on the modulating effects of female sex steroids are reviewed with respect to their effects on ethanol pharmacokinetics. Even though many different methods were used in these studies to manipulate female sex steroids, the results are consistent in suggesting that increases in estrogen inhibit ad lib alcohol consumption. This is true in a broad range of species, including deer mice, rats, monkeys, Sinclair miniature sows, and humans. Available data are consistent with the hypothesis that the mechanism of action may involve impairment of ADH and ALDH via action of estrogen at the liver. More data are needed to resolve unanswered questions.

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Sex-Related Differences in Ethanol-Induced Hypogonadism and Sex Steroid-Responsive Tissue Atrophy: Analysis of the Weanling Ethanol-Fed Model Using Epidemiologic Methods

Judith S. Gavaler

INTRODUCTION

Hypogonadism is a frequent clinical finding in chronic ethanol abusers of both sexes (Cicero 1982; Van Thiel and Gavaler 1982). In the male it is manifested by testicular atrophy, reduced fertility, and impotence. Seminal fluid abnormalities, reduced testosterone concentrations, and inappropriately low gonadotropin levels are common. Although less extensively studied, hypogonadism in the female echoes the findings in the male. Menstrual irregularities, ovulatory inhibition or failure, decreased gonadal mass due to an absence of corpora lutea, and concomitantly reduced progesterone concentrations have been reported to occur in females who are chronically exposed to ethanol.

The ethanol-fed rat model has been widely used to study ethanol-induced reproductive dysfunction. In this model, rats are fed a nutritionally adequate liquid diet, in which 36 percent of the total calories are presented as ethanol, or a control diet, in which dextrimaltose is isocalorically substituted for the ethanol (Lieber 1970). Animals are treated as pairs; each control is pair-fed to be isocalorically matched to its ethanol-fed mate.

We have previously reported single studies using this model. The ethanol-induced hypothalamic-pituitary-gonadal axis disturbances have been reported in rats of both sexes that were started on the diet regimen either as weanlings or as sexually mature adults, as reported by Van Thiel et al. (1975, 1978, 1979) and by Gavaler et al. (1980). In these reports, the differences between the ethanol-fed rats and their individualized isocalorical controls were analyzed with reference to ethanol effects within each sex. The data from these studies, however, have not previously been combined to permit an evaluation of possible differences in susceptibility to ethanol toxicity between the sexes.

METHODS

We have generated data by using the ethanol-fed rat model during the past few years in rats of both sexes that were started on the diet as weanlings. These data have been organized within a data base management system using the computer facilities available at the University of Pittsburgh. As a result, we are now able to examine not only within-sex but also between-sex responses to ethanol exposure. From the weanling-model data, comparable groups from each sex were selected for analysis. We used the ethanol model many more times in males than in females. So as not to exaggerate possible differences between the sexes by using vastly unequal sample sizes, we used all of the female pairs for analysis and selected only those pairs of male rats in which important exposure variables had values similar to those in the females.

Various statistical methods were used with the data. Student's *t*-test was used to test differences of the means of variables in the two groups of ethanol-fed rats, and the paired *t*-test was used to test differences between ethanol-fed animals and their isocaloric controls within each sex. Because the ethanol-fed rat model is a prospective study design, events such as the development of organ atrophy may properly be viewed as incidence; incidence and relative risks were calculated by standard epidemiologic methods (Lilienfeld and Lilienfeld 1980; Fleiss 1981). In this report, atrophy of an organ is defined as occurring when the value obtained as follows is less than 100 percent: the organ:body ratio of the ethanol-fed animal is divided by the same ratio for its isocaloric control and is expressed as a percent. For convenience and consistency, the term atrophy is used throughout; however, for the gonadal data, hypogonadism is a more accurate term. Strength of associations between atrophy rates and ethanol dose levels were evaluated using X^2 corrected for continuity; linear regression (least squares) was used to examine the dose-response data.

RESULTS

Before examining parameters that may be useful in evaluating possible sex-related differences in susceptibility to ethanol toxicity, it is necessary to rule out significant dissimilarities in important ethanol exposure variables that could confound or bias findings of sex-related differences. Indeed, there are no significant differences in the age at which the rats were started on the diet regimen (males: 26.5±0.3 days [mean ±SEM]; females: 25.8±0.4), the age at which the animals were sacrificed (males 75.7±1.1; females: 73.8±0.9), or the duration of ethanol feeding (males: 48.1±0.3; females: 47.6±0.3).

Further, before examining possible susceptibility differences, it is also necessary to determine that previously described differences in weight-related variables have been reproduced in the

samples of pairs of each sex selected for comparison. Differences in body weight (grams) have been replicated: ethanol-fed males (231.7±6.3) weighed significantly less ($p < .05$) than their isocaloric controls (242.7±4.5); similarly, the ethanol-fed females (117.1±4.4) weighed less ($p < .01$) than their controls (133.3±4.3). As in previous reports in weanlings, the ethanol-fed rats consistently weighed less than their isocaloric controls. Therefore, organ weights are corrected for body weight to remove body weight-related differences in organ size for comparisons. As shown in figure 1, the testes:body ratio is significantly lower ($p < .01$) in the ethanol-fed males than in their isocaloric controls; the ovaries:body ratio is similarly reduced in the females ($p < .01$). The seminal vesicles plus prostate:body ratios ($\times 10^4$) are similar in the males (ethanol fed: 28.0±1.8; controls: 26.0±1.3), and the uterus:body ratio ($\times 10^4$) is significantly reduced ($p < .01$)

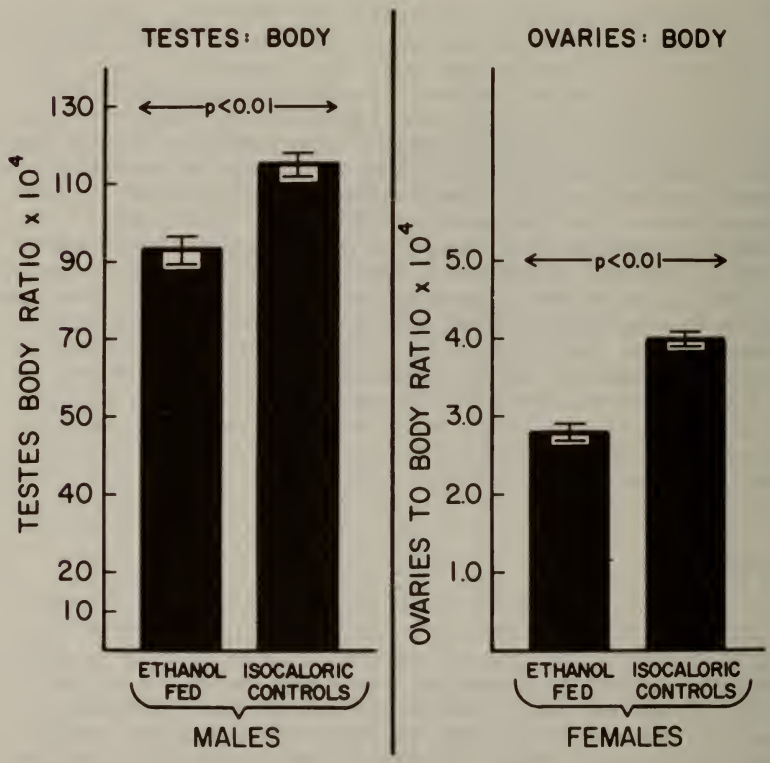

FIGURE 1.--Gonad:body ratio in ethanol-fed rats and isocaloric controls of each sex. Bars represent the mean, and the brackets are SEM values. On each ordinate is the ratio ($\times 10^4$) obtained by dividing the gonad weight (grams) by the body weight (grams) of each animal. The groups of rats are labeled and shown on each abscissa.

in the ethanol-fed females (3.8±0.3) compared with their isocaloric controls (9.1±0.8).

Plasma sex steroid and gonadotropin levels are shown in table 1. Within both sexes, neither follicle stimulating hormone (FSH) nor luteinizing hormone (LH) levels are different. This occurs despite reduced gonadal mass relative to controls in both ethanol-fed groups, as well as despite decreased testosterone levels ($p < .01$) in the ethanol-fed males and reduced progesterone and estradiol concentrations (both $p < .01$) in the ethanol-fed females.

Because the selected groups of ethanol-fed rats of each sex not only reflect previously reported differences when compared with their isocaloric controls but also are comparable with each other in terms of age-related ethanol exposure variables, it is now reasonable to examine the data for possible sex-related differences in susceptibility to ethanol toxicity. Several crude measures that might reveal susceptibility differences are rates of death prior to sacrifice and general atrophy rates of gonads and sex steroid-responsive tissues (uncorrected for ethanol dose). As shown in table 2, neither the death rates nor general gonadal atrophy rates show any difference between the sexes. In contrast, the general uterine atrophy rate is almost two and one-half times higher than the general rate of atrophy of the seminal vesicles plus prostate ($p < .05$). Although these findings are of interest, they lack meaning without some accompanying measure of ethanol dose since the duration of ethanol exposure was the same in both sexes.

The total amount of ethanol calories as well as mean daily ethanol calories were both two times higher in males than in females (both $p < .005$) (table 3). Although these absolute ethanol-intake data are vastly different, before differential susceptibility to ethanol can be considered, a correction for body weight as well as for differences in total body water must be made. Such corrections permit possible sex-related differences to be evaluated in terms of ethanol concentrations (amount per unit volume) rather than in terms of absolute amounts of ethanol. Although total body water demonstrates a signficant sex-related difference in humans, no such difference occurs in rats; therefore, no correction for total body water needs to be made in these rat data (Federation of American Societies for Experimental Biology 1974). In contrast, the difference in body weight of 115 g between the two groups of ethanol-fed animals must be taken into consideration. When the body weight at time of sacrifice is corrected, the ethanol dose data become surprisingly similar. Although the value of the mean daily ethanol calories per gram body weight is higher in the females, the total ethanol calories per gram body weight is identical for both sexes (table 3).

Fortuitously, because the mean values of one of the body-weight-corrected ethanol intake measures are identical in both sexes, atrophy rates at various dose levels can be compared directly. The gonadal atrophy rates as a function of total ethanol calories

TABLE 1.--Sex steroids and gonadotropin concentrations* in the ethanol-fed weanling model

Hormones		Males		Females	
		ETOH-fed	Controls	ETOH-fed	Controls
FSH	(ng/cc)	459.6±67.5	357.7±26.8†	159.0±12.8	181.0±16.2†
LH	(ng/cc)	57.6±5.1	61.5±4.1†	68.7±5.7	79.4±6.8†
Testosterone	(ng/cc)	3.1±0.4	5.0±0.7‡		
Progesterone	(ng/cc)			23.3±4.3	54.3±7.3‡
Estradiol	(pg/cc)	30.1±3.3	27.6±3.2†	27.5±1.2	33.3±1.5‡

*Mean ±SEM.

†No significant difference in ETOH-fed compared with isocaloric controls.

‡p <.01 ETOH-fed compared with isocaloric controls.

TABLE 2.--Relative risks in weanling-model rats without regard for ethanol dose

Rate*	Males	Females	Relative risk†	X^2	Significance
Death prior to time of sacrifice	5.8	5.9	0.98	~0	NSD
Gonadal atrophy	75.7	74.4	0.95	~0	NSD
Sex steroid-responsive tissue atrophy	35.1	87.2	2.48	4.49	$p < .05$

$$*\text{Rate} = \frac{\text{number of rats in which the event occurred}}{\text{number of rats at risk for the event to occur}} \times 100$$

$$\dagger\text{Relative risk} = \frac{\text{rate in female ETOH-fed rats}}{\text{rate in male ETOH-fed rats}}$$

TABLE 3.--Ethanol intake data in weanling-model rats*

Ethanol variable	Males	Females
Total ethanol calories	650.6±20.3	340.5±16.3†
Mean daily ethanol calories	13.3±0.2	7.2±0.2†
Total ethanol calories per gram body weight	2.9±0.1	2.9±0.1
Mean daily ethanol calories per gram body weight	0.058±0.001	0.062±0.001‡

*Mean ±SEM.

†p <.005.

‡p <.05.

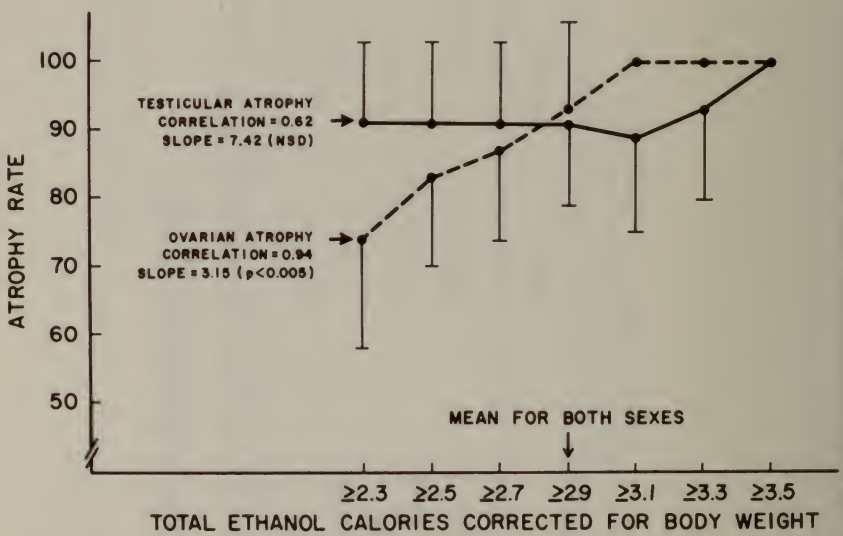

FIGURE 2.--Gonadal atrophy rates in ethanol-fed rats of each sex. On the ordinate is the atrophy rate (×100), where the number of rats in which atrophy has occurred is divided by the number of rats at risk of developing atrophy; at each dose level, those rats that have already developed atrophy at a lower dose level are no longer at risk and are therefore in neither the numerator nor the denominator. Each rate is shown with its 95 percent confidence interval. On the abscissa are total ethanol calories per gram body weight; the range shown is the mean ±1 SD.

per gram body weight are shown in figure 2. Although the testicular and ovarian atrophy rates per se are not significantly different from each other at any point on the dose-response curve, several observations about these data are pertinent. The ovarian atrophy rates show a linear response not only with a high correlation (0.94) but also with a slope that is significantly different from zero. This fact can be interpreted as showing a statistically significant dose response to increasing ethanol concentrations. In contrast, the testicular atrophy curve is statistically flat--i.e., the slope is not statistically different from zero; this fact can be interpreted as indicating a lack of association between the dose level and the incidence of testicular atrophy. Finally, the gonadal atrophy rates in both sexes are high. Considering only those rates that are within one standard deviation (SD) of the mean [2.9±0.6 (mean ±SD) total ethanol calories per gram body weight] as shown in figure 2, both sexes have rates in excess of 90 percent (males: 93±4; females: 91±10).

In contrast to the gonadal atrophy findings, the sex steroid-responsive tissue atrophy rates demonstrate significant differences between the sexes over the entire dose curve.

As shown in figure 3, the uterine atrophy rates are significantly and consistently higher than those for the seminal vesicles plus prostate. Further, as with the gonadal atrophy rates, although the uterine rates show a statistically significant dose response, the seminal vesicles plus prostate do not.

DISCUSSION

The rationale for examining the possibility of sex-related differences in reproductive system susceptibility to ethanol toxicity is twofold. First, the question of whether a differential susceptibility between the sexes exists has previously been raised with reference to the toxic effects of ethanol on the liver. The major publications that have suggested that females are more susceptible than males to alcoholic hepatitis and ethanol-induced cirrhosis have been reviewed recently (Gavaler 1982). Taken as a whole, these studies support the hypothesis of sex-related differential susceptibility. Second, the fetal alcohol syndrome (FAS) has been documented. The fact that it exists suggests either that the actual mechanisms for, and/or the expression of, hypogonadism are dissimilar in males and females or that endocrine/reproductive system dose responsiveness to ethanol differs between the two sexes or a combination of these two alternatives.

Although exploratory, the results of this analysis of possible sex-related differences in ethanol-induced hypogonadism and sex steroid-responsive tissue atrophy suggest that differences between the sexes do exist. The present analysis deals strictly with dichotomized outcomes--i.e., the presence or absence of hypogonadism or sex steroid-responsive tissue atrophy. These results

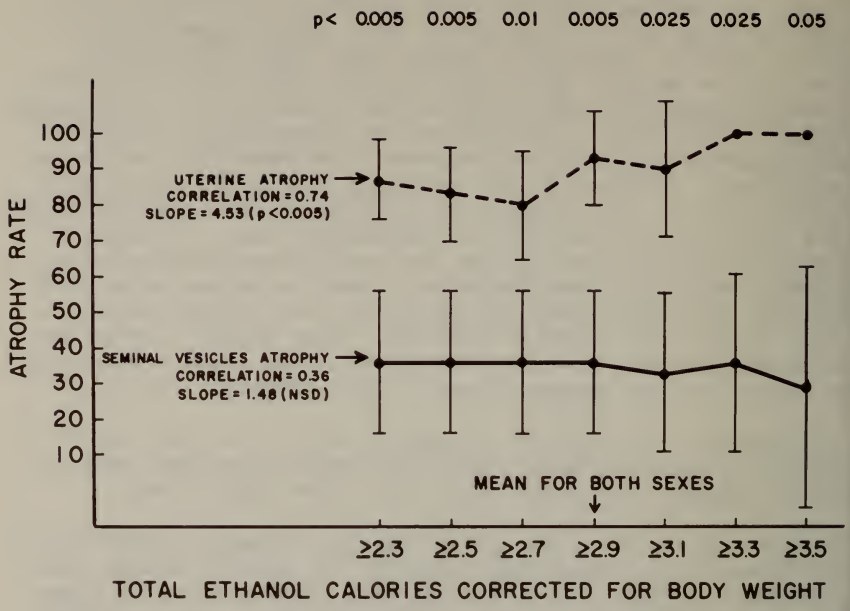

FIGURE 3.--Sex steroid-responsive tissue atrophy rates in ethanol-fed rats of each sex. On the ordinate is the atrophy rate (× 100); each atrophy rate is shown with its 95 percent confidence interval. On the abscissa are total ethanol calories per gram body weight; the range shown is the mean ±1SD. The p values above the two curves indicate the level of significance of the difference in atrophy rates in the two sexes at that dose level.

can provide no information about the degree of hypogonadism or atrophy either between the sexes or within sexes at various levels of ethanol exposure. The data support two broad conclusions: (1) the seminal vesicles plus prostate may be more resistant to ethanol toxicity than the uterus; (2) there appears to be no qualitative difference between the sexes in rates of hypogonadism development. However, the analysis suggests that the characteristics of the quantitative dose response, as manifested in hypogonadism incidence, may differ between the sexes. We do not know whether these findings can be applied to and/or reproduced in rats that are fed for longer or shorter times and started on the diet regimen not only at earlier or later ages, but also as adults.

Are there characteristics in rats of each sex that differ between those with and those without hypogonadism or sex steroid-responsive tissue atrophy? Exogenous estradiol has been shown

to be capable of maintaining ventral prostate mass in castrated male rats (Corrales 1981); although the mean level of testosterone was decreased in the ethanol-fed males compared with their controls, the mean concentrations of estradiol were similar (table 1). It is possible that such levels of estradiol in the ethanol-fed males were sufficient, on mean, to maintain seminal vesicles plus prostate in these rats. Clearly, the testosterone and estradiol levels should be analyzed in relation to the presence or absence of hypogonadism or atrophy of seminal vesicles plus prostate. Further, is there an interaction between time and dose; do rats of one sex develop atrophy at an earlier point on the dose and time curve than the other sex? These and many other questions from the present data, which support a hypothesis of sex-related differences in reproductive/endocrine system susceptibility, merit further attention.

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Hypophyseal Hormones and Ethanol: Genetic Studies in Mice

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INTRODUCTION

ETHANOL AND HORMONES

Ethanol exerts multiple effects on endocrine functioning. In some instances, acutely administered ethanol may directly influence endocrine tissue. For example, Cicero et al. (1980) reported that ethanol inhibited testicular steroidogenesis in vitro in dispersed cell preparations of rat testes. Acute ethanol elevates cortisol levels in humans (Ylikahri et al. 1978), and this may not be accompanied by an increased release of ACTH (Leppaluoto et al. 1975), which suggests a direct effect on the adrenals (Cobb et al. 1981). Endocrine effects may also develop after chronic exposure to ethanol. Many alcoholics apparently have selective ACTH deficiency (Merry and Marks 1969), and a pseudo-Cushing's syndrome, which is difficult to distinguish from true Cushing's syndrome, has been reported in alcoholics (Rees et al. 1977). Several recent reviews have covered the different endocrine effects of ethanol (Farmer and Fabre 1975; Wright 1978; Van Thiel 1980; Ylikahri et al. 1980).

It is of paramount importance to determine whether endocrine effects of ethanol are critically related to the development of physical dependence on the drug. Most studies designed to address this question necessarily use some animal model of alcoholism. One approach has been to manipulate the endocrine status of animals during the induction of tolerance to and physical dependence on ethanol. Such studies are reviewed by Crabbe and Rigter (1980). For example, Sze and coworkers (1974) reported that by adrenalectomizing mice before inducing physical dependence on ethanol with a liquid diet, they prevented the ensuing seizures when ethanol was withdrawn. They interpreted

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this as a "permissive" role for glucocorticoids in the development of ethanol dependence. Wood (1977) reported that chronic dexamethasone treatment speeded the development of tolerance in rats that were treated chronically with ethanol. Chronic blockade of glucocorticoid receptors with cortexolone exerted the opposite effect (i.e., a blockade of tolerance) in mice that were chronically treated with ethanol (Tabakoff and Yanai 1979).

ETHANOL AND GENETICS

Alcoholism has a clearly familial pattern of incidence, and much evidence (reviewed by Goodwin 1980) supports a genetic contribution to susceptibility to alcoholism. In animal studies, the importance of genotypic differences in the determination of sensitivity to ethanol, tolerance development,and susceptibility to physical dependence is receiving much recognition. Investigators who are studying the biochemical and physiological bases of alcoholism now generally believe that genotype can influence all these different aspects of sensitivity to the effects of ethanol. A recent review by Crabbe and Belknap (1980) considered the evidence for genotypic mediation of ethanol's effect in animals. One conclusion was striking: although susceptibility to almost all aspects of ethanol's effects in animals is clearly inherited to some degree, there is little idea yet of what is inherited. There is even less information about the relationships among different responses to ethanol at the genetic level. For example, it is not known whether individuals who are genetically very susceptible to developing physical dependence on ethanol differ in their rate or degree of tolerance development.

ETHANOL, HORMONES, AND GENETICS

Because of genotype influences on virtually all aspects of ethanol sensitivity, tolerance, and physical dependence, it seems likely that individual differences in endocrine responses to ethanol will have some genetic component as well. Few studies have explored the role of genetic differences in endocrine responsiveness to ethanol. Kakihana et al. (1968) administered acute ethanol to several inbred strains of mice and reported variable degrees of adrenocortical activation that correlated with the degree of sensitivity to the hypnotic effect of ethanol in the strains. Kakihana (1979) later reported that corticosterone levels were elevated during the development of physical dependence, and they were elevated further during withdrawal in DBA/2 mice that were fed a liquid diet containing ethanol, but there were no increases in C57BL/6 mice. In the studies reviewed below, we first document genotypic differences in pituitary content of the three hormones in several inbred strains of mice. We also report genotypic variation in the severity of physical dependence on ethanol. Further, we document genotypic variability in the effects on the pituitary-adrenal axis of physical dependence on ethanol and the withdrawal from physical dependence.

MATERIALS AND METHODS

ANIMALS

Male, inbred mice were purchased at the age of 4 to 5 weeks.

INDUCTION OF PHYSICAL DEPENDENCE

Mice were rendered physically dependent on ethanol in inhalation chambers according to a method detailed elsewhere (Crabbe et al., in press). Mice were housed in wire-mesh cages suspended inside a large plastic (Pleixglas) chamber. Ethanol vapor concentrations were determined by gas chromatography and varied no more than 0.2 mg ETOH/L air when samples were drawn from different locations in the chamber. Mice were weighed and injected with a priming dose of ethanol (1.5 g/kg, 20 Δv/v in physiological saline). A 1.0-mol/kg injection of pyrazole hydrochloride was given intraperitoneally (IP) (0.1 cc/kg). Pyrazole inhibits ethanol metabolism and results in stable blood-ethanol levels when used in conjunction with an inhalation system. The advantages and disadvantages of this method were reviewed recently (Goldstein 1980). At 24 and 48 hours later, mice were briefly removed from the chamber, and an additional pyrazole injection was given.

ASSESSMENT OF WITHDRAWAL FROM DEPENDENCE

Physical dependence on ethanol must be inferred from the severity of symptoms assessed after ethanol is withdrawn. The most striking sign of withdrawal is the handling-induced convulsion (HIC). Mice were scored hourly for HIC according to a system that was modified slightly from Goldstein and Pal (1971). We have published the rationale for this system (Crabbe et al. 1980). Mice were removed from the chamber at 8 a.m. (after 72-hours exposure and 24 hours after the last pyrazole injection), and they were scored hourly until withdrawal signs clearly subsided. The area under the withdrawal curve was analyzed.

EXTRACTION OF PEPTIDES FROM PITUITARY

Animals were sacrificed by decapitation, and their pituitary glands were rapidly removed and placed in the homogenization-extraction buffer (5N acetic acid containing 1mM each of the proteolytic enzyme inhibitors phenylmethyl-sulfonyl fluoride and iodoacetamide. The sample was homogenized in a hand-driven polytetrafluoroethylene (Teflon) tissue homogenizer, followed by three cycles of freezing and thawing (dry ice and 37°C). After homogenization, the tissue extract was centrifuged, and the supernatant was removed and frozen until radioimmunoassay (RIA).

RADIOIMMUNOASSAYS

RIA of plasma corticosterone from a tail-vein blood sample was performed as previously described (Keith et al. 1978). Peptide RIA tests were done as previously described (Rees et al. 1971). The ACTH antiserum used has midportion (11-24 ACTH) specificity. The β-endorphin antiserum used cross-reacts on an approximately equimolar basis with β-LPH and β-endorphin. This antiserum recognizes all the modified forms of β-endorphin (61-91 β-LPH) but does not cross-react with Met- or Leu-enkephalin. Both antisera recognize the common precursor pro-opiomelanocortin (POMC). The α-MSH (melanophore stimulating hormone) antiserum used does not cross-react with 1-39 or 1-24 ACTH, and has only a slight cross-reactivity with 1-10 ACTH. This antiserum does not recognize POMC. The compounds β_h-(1-39)ACTH, (C.H. Li, Hormone Research Laboratory, Berkeley, California), β_h-endorphin, and α-MSH (Peninsula Laboratories) were used as standards in the RIA tests, as well as for iodination.

RESULTS AND DISCUSSION

GENOTYPIC DIFFERENCES IN HYPOPHYSEAL HORMONES

Resting pituitary content in four to seven male mice of each of 16 highly inbred strains was determined by RIA with antisera for three hypophyseal hormones. Strains differed significantly in pituitary content of α-MSH ($F_{15,83} = 6.55$, $p < .01$, see figure 1). Strain differences of comparable magnitude were found for β-endorphin ($F_{15,83} = 7.09$, $p < .01$) and ACTH immunoreactivity ($F_{15,83} = 17.70$, $p < .01$). We conclude that there is significant genetic influence on the resting content of these hormones. Since α-MSH is strictly located in the intermediate lobe, endorphin is located in both anterior and intermediate lobes, and ACTH is virtually all located in anterior lobe, it was of interest to see if the same genes influenced all three of these hormones or if they were under independent genetic control. We calculated the correlations among the mean values for the inbred strains to estimate the genetic correlation coefficient, according to the procedure suggested by Hegmann and Possidente (1981). β-Endorphin and α-MSH content correlated $r = .62$ ($p < .01$), β-endorphin and ACTH content correlated $r = .94$ ($p < .01$), and α-MSH and ACTH content correlated $r = .55$ ($p < .05$). The high correlation between ACTH and β-endorphin is probably largely due to the equimolar recognition of the common precursor by both these antisera. However, the α-MSH antiserum does not recognize POMC, so the correlations between α-MSH and the other hormones may reflect the influence of common genes to some degree. Because we did not weigh the pituitary glands, it is possible that the overall pattern of asssociation among content measurements was secondary to differences in pituitary size. Previously published preliminary

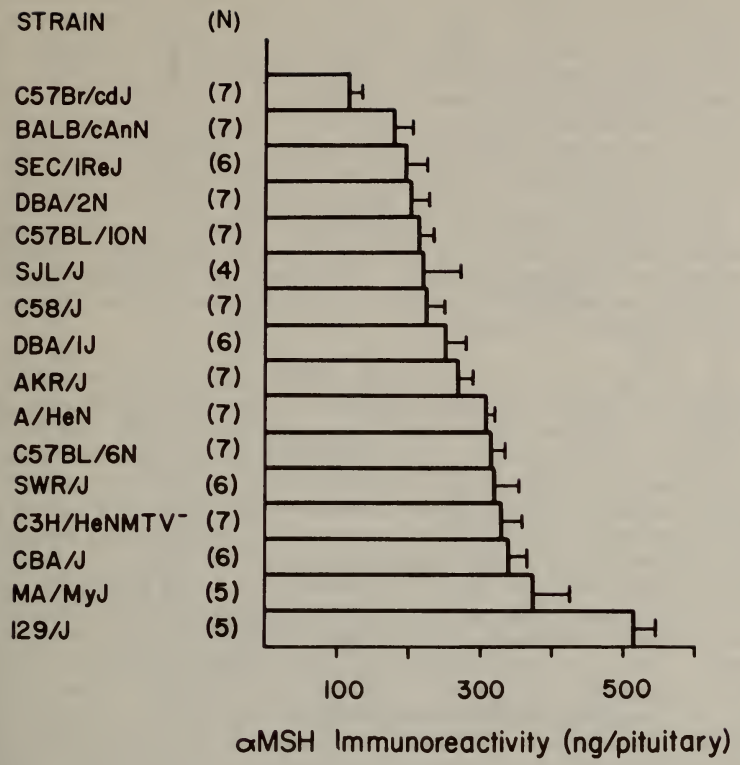

FIGURE 1.--α-MSH immunoreactivity for different strains of mice

determinations of β-endorphin and ACTH content in five inbred strains revealed strain differences of similar magnitude that were independent of pituitary weight. Thus, we believe that this is unlikely to account for all the strain differences we found (Crabbe et al. 1981).

GENOTYPIC DIFFERENCES IN ETHANOL-WITHDRAWAL SEVERITY

We have recently reported that the inbred strains of mice studied here varied significantly in their degree of physical dependence as shown by the severity of HIC during withdrawal (Crabbe et al., unpublished data). To see if there was any relationship between the strain differences in pituitary hormonal functioning as reflected in the levels reported above and ethanol-withdrawal severity, we estimated the genetic correlations from the mean strain values as described above. β-Endorphin content correlated

with the area under the HIC withdrawal curve $r = .37$ ($p = .18$), ACTH content correlated $r = .41$ ($p = .13$), and α-MSH content correlated $r = .54$ ($p = .04$). Since all strains were subjected to the same dose of ethanol vapor, and strains differed significantly in blood-ethanol levels achieved during dependence induction, we corrected the HIC withdrawal area index for the differences in blood-ethanol concentration at the time of withdrawal and calculated the partial correlation coefficients of withdrawal severity and hormone contents. None of the three correlations was significantly affected. Thus, we tentatively conclude that genetically determined differences in pituitary hormonal content were influenced to some degree by the same genes that were influencing ethanol-withdrawal severity. This led us to experiments designed to see whether different strains varied in the effects of ethanol on pituitary hormones.

Mice of five inbred strains (AKR/J, BALB/cAnN, C3H/HeNMTV-, C57BL/6N, and DBA/2N) were rendered physically dependent on ethanol for 3 days as described. Separate groups of 7 to 12 mice were treated with pyrazole or saline but were not given ethanol. After 72 hours of inhalation, all mice were sacrificed, and the pituitary content of β-endorphin was determined. In all strains except DBA/2N, the endorphin content was significantly reduced by approximately 30 to 50 percent ($ps < .01$). Saline and pyrazole control groups did not differ, indicating that the depletion was specific to ethanol treatment. Separate groups of mice were withdrawn to ensure that all strains were physically dependent on ethanol. Thus, strains differed in their susceptibility to endocrine manipulation by ethanol dependence induction. We then sought to determine whether there would be further changes in endocrine functioning, indicated by altered levels of β-endorphin during withdrawal from ethanol dependence. Animals were sacrificed 24 hours after withdrawal from ethanol dependence, and the endorphin content was significantly elevated in the AKR/J, BALB/cAnN, and C57BL/6N strains ($ps < .01$). No changes were seen in the other two strains. In the AKR/J strain, content was not elevated in either pyrazole or saline control groups, while in the BALB/cAnN and C57BL/6J strains, the pyrazole-treated group also showed elevated content. We conclude that ethanol dependence and withdrawal exerted genotype-dependent changes in pituitary status. We next sought to characterize further the generality and time-dependence of these changes in the AKR/J strain.

We rendered AKR mice dependent on ethanol as described above and assayed pituitary content at different times during withdrawal. Separate saline-treated and pyrazole-treated groups were treated and assayed similarly, except that they were not given ethanol. Figure 2 shows the changes in β-endorphin content at different times during withdrawal. β-Endorphin content was not significantly reduced when the animals were withdrawn from ethanol as we had previously found. However, there was a tendency ($p < .10$) for a significant reduction in content at 6 hours after withdrawal and a highly significant decrease ($p < .01$) in content

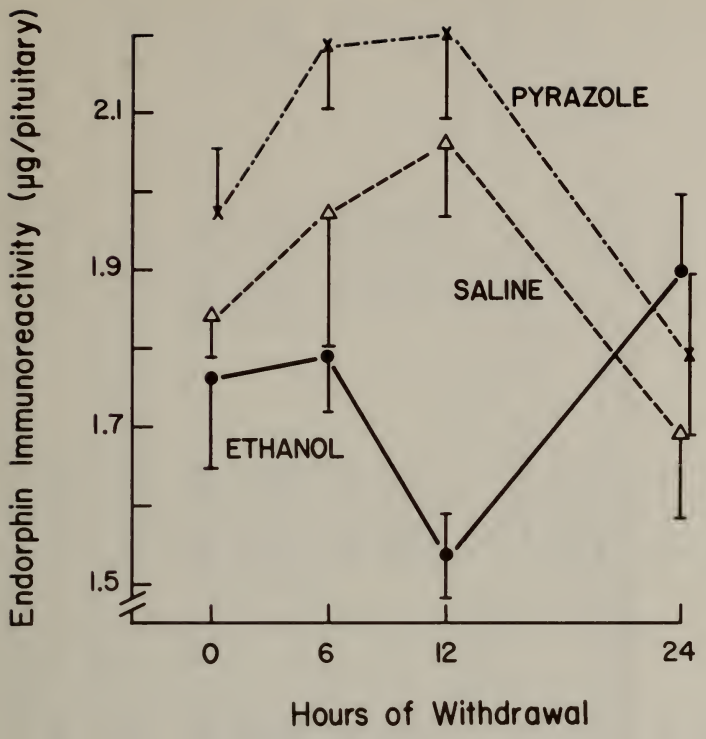

FIGURE 2.--Changes in β-endorphin content during ethanol withdrawal for AKR mice

at 12 hours after withdrawal. This finding corresponds to the point at which maximal withdrawal seizures are seen. By 24 hours after withdrawal, when withdrawal seizures had almost completely subsided, there was no difference among the groups. A similar pattern of changes was seen in α-MSH content (see table 1). ACTH content showed a different temporal pattern of changes. ACTH content tended to be reduced at the time of withdrawal ($p < .10$), but there were no further significant differences during withdrawal (see table 1). Consistent with the report of Kakihana (1979), plasma corticosterone levels in these mice were significantly elevated at the time of withdrawal and showed further elevations as withdrawal progressed, at both 6 and 12 hours ($ps < .01$). Corticosterone levels were again not different from controls at 24 hours after withdrawal.

Finally, we attempted to chart the progress of endocrine changes during the development of ethanol dependence in AKR/J mice (table 2). As ethanol dependence developed, α-MSH content

TABLE 1.--Changes in plasma corticosterone and in pituitary content of α-MSH and ACTH during withdrawal from physical dependence on ethanol (mean and SEM)

		Hours after withdrawal			
		0	6	12	24
α-MSH (ng/pituitary)	Dependent withdrawn	345 (16)	380 (12)	262 (28)	454 (17)
	Combined controls	418 (29)	430 (28)	454 (15)	412 (20)
ACTH (ng/pituitary)	Dependent-withdrawn	1,017 (36)	1,191 (41)	1,071 (56)	1,093 (31)
	Combined controls	1,151 (26)	1,281 (39)	1,126 (21)	1,121 (41)
Plasma corticosterone (ng/ml)	Dependent-withdrawn	165 (26)	263 (23)	225 (43)	86 (16)
	Combined controls	34 (04)	78 (10)	92 (12)	62 (08)

TABLE 2.--Changes in plasma corticosterone and in pituitary content of β-endorphin, α-MSH, and ACTH during the development of physical dependence on ethanol (mean and SEM)

		Hours of exposure to ethanol			
		0	24	48	72
β-Endorphin (ng/pituitary)	Dependent	1,897 (78)	1,659 (77)	1,577 (55)	2,313 (105)
	Combined controls	2,006 (54)	1,941 (50)	1,818 (61)	2,228 (77)
α-MSH (ng/pituitary)	Dependent	447 (18)	329 (15)	329 (16)	403 (15)
	Combined controls	458 (12)	378 (12)	379 (10)	460 (16)
ACTH (ng/pituitary)	Dependent	1,182 (61)	1,198 (39)	1,389 (43)	1,204 (48)
	Combined controls	1,150 (28)	1,199 (62)	1,367 (31)	1,358 (47)
Plasma corticosterone (ng/ml)	Dependent	45 (09)	56 (22)	76 (06)	133 (10)
	Combined controls	41 (05)	56 (06)	49 (05)	61 (07)

underwent transient depletions, significant ($p < .05$) after both 24 and 48 hours of inhalation. β-Endorphin content was significantly ($p < .01$) depleted at 24 hours. By 72 hours, the usual time of withdrawal, neither hormone was significantly depleted. ACTH content again followed a different pattern, showing no difference until 72 hours of treatment, when there was measurable depletion ($p < .05$). Plasma corticosterone levels corresponded well with the ACTH levels in the pituitary, beginning to increase after 48 hours of treatment. Plasma corticosterone was significantly elevated after 72 hours of treatment ($p < .01$; see table 2).

These differences may be compared with those reported by Schulz et al. (1980) and Gianoulakis et al. (1981). The former found reductions in intermediate and anterior lobe content of β-endorphin in rats that were chronically treated with ethanol in their drinking water. The latter authors intubated rats with ethanol daily and found increased incorporation of radiolabeled phenylalanine into intermediate lobe endorphins, increased release into incubation medium but no depletion of content in intermediate lobe tissue. In neither experiment were the animals demonstrably physically dependent on ethanol. A recent report (Genazzini et al. 1982) identified large depletions in cerebrospinal fluid (CSF) levels of β-endorphin but not β-LPH in alcoholics who were undergoing withdrawal. CSF levels of ACTH were elevated. None of those changes were seen in plasma.

Our results demonstrate the existence of genotypic differences in hypophyseal hormone functioning as reflected in differences in resting levels of three hormones. Genotypic differences in susceptibility to physical dependence on ethanol were also apparent. The existence of genetic correlations between strain values of these parameters suggests that there may be an important degree of common genetic determination. The effect of ethanol on pituitary hormone levels also varies genotypically during both the development of physical dependence and the withdrawal. Within the AKR/J strain, the time course of development of endocrine differences is specific to the hormone studied. We cautiously suggest that there may be differential effects of ethanol on pituitary hormones of intermediate lobe versus anterior lobe origin and that these differences may be strain dependent.

ACKNOWLEDGMENT

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Effect of Alcohol on Aldosterone Metabolism

Louis F. Fabre, Jr.

INTRODUCTION

In the mid-1960s, it seemed that physicians needed a simple urine or blood test that would differentiate the alcoholic from the average person who occasionally drinks too much. My group consisted of experts in ultramicroanalysis who could measure minute quantities of chemical compounds in body fluids. During those years, gas chromatographic methods were developed for measuring steroids such as cortisol (Fabre et al. 1969b), aldosterone (Fabre et al. 1969c), testosterone, and so forth. Later radioimmuoassay (RIA) procedures were developed to measure these compounds (Farmer et al. 1972, 1973) and others, such as insulin (Farmer and Fabre 1974b). As the methodology was developed, clinical experiments were devised to test the effect of alcohol consumption on endocrine systems in humans. Unfortunately, the simple test for alcoholism was never developed; however, a great deal of data concerning the effects of alcohol on endocrine systems was accumulated.

Salt and water balance has always intrigued investigators of alcoholism. Early literature had shown that alcohol consumption inhibits antidiuretic hormone secretion, resulting in diuresis (Eggleton 1942). Curiously, in alcohol-withdrawal states, both animals and humans seemed overhydrated (Beard et al. 1965) rather than underhydrated.

Many of us have probably had too many drinks on occasion and found ourselves urinating frequently. The next morning we awake very thirsty, but we might notice some puffiness around our eyes or maybe some swelling of our feet. The scales confirm that a few pounds have been gained. As the day goes on, the thirst diminishes, the puffiness disappears, and by the next day the extra pounds are lost. This is a common example of the effect of alcohol on aldosterone secretion and thus on salt and water balance.

The problems, of course, are that: (1) alcohol affects many systems besides aldosterone; (2) the effects are different in sobriety, alcoholism, the social drinker, and the reformed alcoholic; and (3) different results occur with rising alcohol blood

levels, steady-state blood levels, and declining blood levels, leaving a great many permutations and combinations. All these variables make it difficult to study a system with dynamic equilibrium characteristics, such as aldosterone. Unfortunately, only a few of the critical experiments were performed in the early years. Those are summarized here.

MATERIALS AND METHODS

The initial studies were performed on dogs. As further information became available, studies were done on abstaining male alcoholic subjects and in normal healthy male volunteers. The latter two groups were given alcoholic beverages under clinical metabolic laboratory situations.

CANINE STUDIES

Male dogs, weighing 20 to 30 kg, were housed in air-conditioned kennels and maintained on nutritious dog chow. A conditioning period of at least 14 days before experimentation was used to eliminate possible errors due to differing states of nutrition.

The animals were anesthetized with sodium pentobarbital with an initial dose of 30 mg/kg; no supplemental anesthesia was given. The femoral artery and vein were cannulated to monitor blood pressure and administer infusions.

Three different experimental techniques were used. In the original experiments, 0, 24, or 48 ml of ethanol were infused over a 2-hour period in 250 ml of normal saline. During the infusion period, the animals were hemorrhaged via the lumboadrenal vein, permitting total collection of left adrenal venous outflow. Aldosterone and hydrocortisone in this total sample were isolated by paper chromatography and measured by ultraviolet absorption (Farrell et al. 1956). Blood ethanol was not measured.

In the second set of experiments, ethanol was administered in 250 ml of saline (11 ml/kg) over a 1-hour period in concentrations up to 30 percent. Ethanol dosage was calculated before the experiment to achieve a blood-ethanol level of 300 mg/100 ml. Serial blood-ethanol determinations were performed to quantitate the alcohol level and ensure its constancy. After the initial infusion, the blood-ethanol concentration was maintained by infusion of 25 percent ethanol in saline at approximately 12 ml/hour. The lumboadrenal vein was cannulated, and adrenal effluent was collected in serial 15-minute fractions. Aldosterone was isolated and quantitated by a gas chromatographic method (Fabre et al. 1969c); hydrocortisone was isolated by thin-layer chromatography with methanol as the eluent and quantitated colorimetrically.

In a third set of experiments, a lower dose of ethanol was used. The animals were infused to establish a blood level of 75 to 250 mg percent, and the same parameters as described above were quantitated.

HUMAN STUDIES

The subjects were all abstaining chronic alcoholic volunteers between the ages of 25 and 45. They were well screened and found to be in good health (Mendelson and La Dou 1964). During all phases of the experiment, the subjects were maintained on a well-balanced diet that was supplemented with vitamins. Water was consumed ad lib, and no sedative, tranquilizer, or hypnotic drug was administered.

In the first set of experiments, daily, consecutive 24-hour control urine specimens were obtained for 5 days. On day 6 and continuing for 14 days, the subjects were allowed ethanol ad lib (a standard brand of bourbon). Thereafter, the subjects were abruptly withdrawn from ethanol and studied for an additional 5 days. Urinary aldosterone excretion was measured by a modification of a gas chromatographic method (Bravo and Travis 1967). Throughout the experiment, blood-ethanol determinations were estimated by a Breathalyzer technique.

A second set of experiments was performed in which aldosterone secretory rates were measured (Flood et al. 1961). Each subject was used as his own control. During the control period, the subjects were injected with 9 μCi of H^3-aldosterone at 8:00 a.m., and consecutive 6-hour urines were collected for 48 hours. After 1 week, the volunteers were allowed ethanol ad lib; 24 hours later, at 8:00 a.m., 9 μCi of H^3-aldosterone were injected, and consecutive 6-hour urine specimens were obtained.

In a third set of experiments, five normal young males (nonalcoholic), 21 to 25 years, on an ad lib diet, were maintained supine between 6:00 a.m. and 6:00 p.m. on each of 2 days (Farmer and Fabre 1974a). On day 1, orange juice was given between 8:00 a.m. and 12:00 noon. On day 2, during the successive hours beginning at 8:00 a.m., vodka was administered hourly in 5-, 3-, 2-, 2-, and 2-oz quantities mixed with orange juice. Blood samples were tested for plasma aldosterone, cortisol, and renin activity. The fourth set of experiments on chronic alcoholic subjects was the same as the first set except that plasma cortisol, plasma renin activity, and urinary Na/K ratio were also measured.

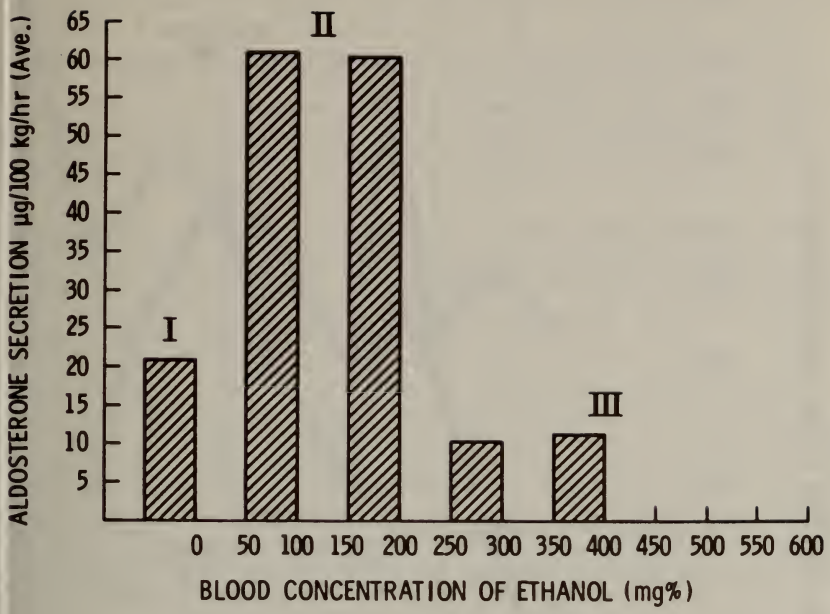


FIGURE 1.--Dose-response relationship for aldosterone secretion versus blood-ethanol concentration; group I, controls; group II, low-dose ethanol; group III, high-dose ethanol

RESULTS

CANINE STUDIES

The results of the animal studies are presented in figures 1 and 2. Figure 1 shows the dose-response relationship of ethanol to adolsterone secretion. If the experiments are divided into three groups according to blood-ethanol level, one can see the following: (1) the controls secreted aldosterone at a rate of approximately 21/mg/100kg/hour; (2) animals that received low doses of ethanol (blood-ethanol concentrations of 50 to 200 mg percent) secreted aldosterone at a rate of 60 mg/100kg/hour; and animals that received high doses of ethanol (greater than 250 mg percent blood ethanol level) secreted aldosterone at a rate of 10 mg/100kg/hour or less. Thus, low doses of ethanol stimulate aldosterone secretion, and high doses of ethanol inhibit it (Fabre et al. 1971).

To present the data in another way, figure 2 shows the sequential aldosterone secretory rates throughout the experiment. Normal control dogs show the typical rise in aldosterone secretion

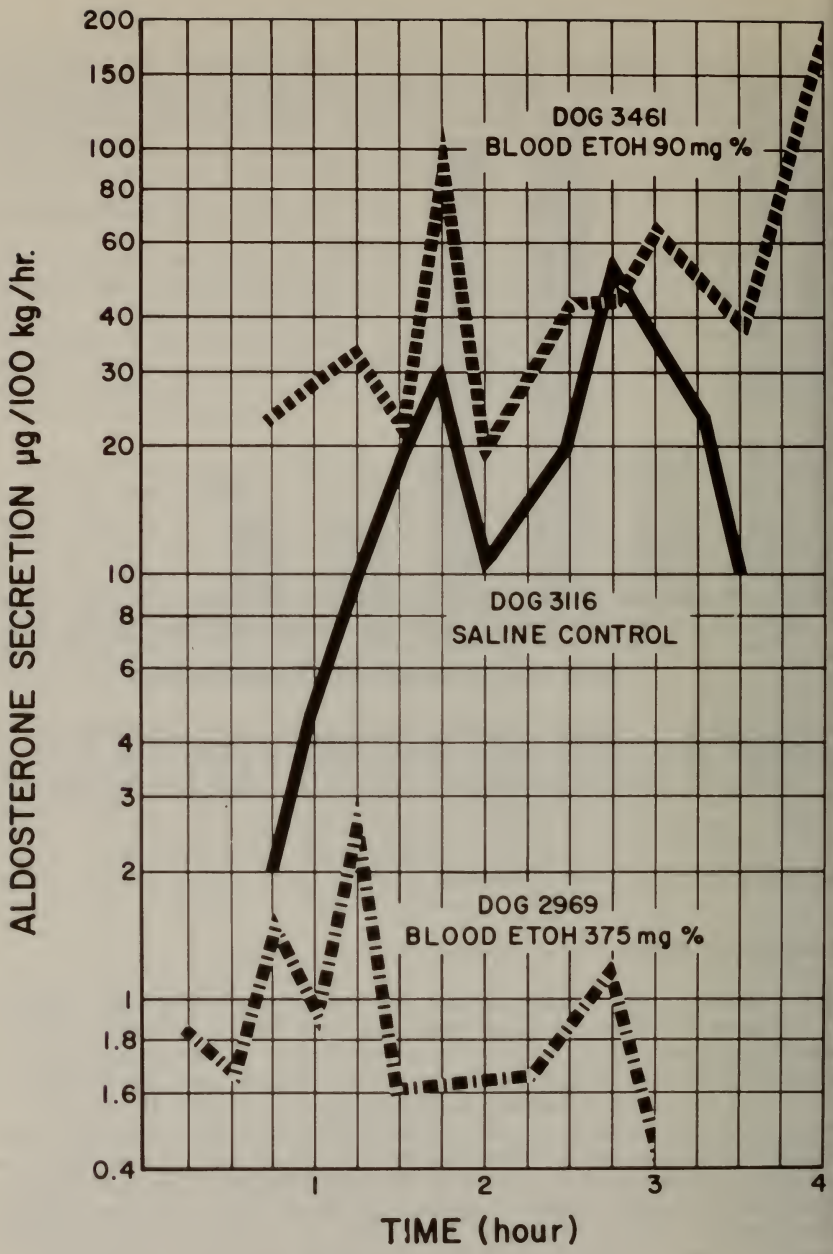


FIGURE 2.--Sequential aldosterone secretory rates for a control animal (dog 3116), animal receiving a high dose of ethanol (dog 2969), and animal receiving a low dose of ethanol (dog 3461) (note semilog scale)

as a result of hemorrhage. Low-dose ethanol animals start secreting aldosterone at a higher rate and respond to hemorrhage with a greater increase in aldosterone secretion. High-dose ethanol animals start secreting aldosterone at lower levels and have a blunted response to hemmorrhage. The results are so clear that when they are plotted, only one overlap occurs throughout the entire data-collection period. The results are statistically significant when they are analyzed by analysis of variance, $p = .01$ (Fabre et al. 1972b).

Ethanol also affected survival time to hemorrhage (figure 2). The control dog lived 3.5 hours, the low-dose ethanol dog 4.0 hours, and the high-dose ethanol dog 3.0 hours. Adrenal blood flow and mean arterial blood pressure were also altered in the ethanol-treated groups (Fabre et al. 1972a).

Hemorrhage is a potent stimulus to aldosterone secretion and produces near-maximum aldosterone secretory rates (Farrell et al. 1956). However, the low-dose ethanol-treated animals produced aldosterone secretory rates that were higher than any previously seen--up to 200 mg/100kg/hour. As stated, the high-dose ethanol-treated animals did not respond to hemorrhage with increased aldosterone secretion. In figure 2, dog 2969, the high-dose animal, had only one collection period that equaled the starting adolsterone secretory rate of control dog 3116. Therefore, aldosterone secretion in the pentobarbital-anesthetized hemorrhaged dog seems to be profoundly altered by alcohol, with stimulation occuring with blood-alcohol levels in the range of 50 to 200 mg percent and inhibition occurring with alcohol levels greater than 300 mg percent.

HUMAN STUDIES

In the first set of experiments, 24-hour urine specimens were obtained before and after ad lib consumption of beverage alcohol. The control value was arbitrarily considered to equal 100 percent. On day 1, a 400 percent increase in aldosterone excretion was observed. On day 2 of drinking, the increase over the control value was 500 percent (figure 3). Further elaboration of these results in figure 4 reveals that with each individual serving as his own control, for 13 subjects the average control aldosterone excretion was 2.66 mg/6 hours and the average aldosterone excretion 24 hours after ad lib ethanol consumption was 4.97. The difference (2.35 mg/6 hours) is significant: $p = .02$ (paired t-test) (Fabre et al. 1971).

Sequential aldosterone excretory rates and blood-ethanol levels for one typical individual are shown in figure 5. Control aldosterone excretory rates during the first 5 days in the experiment ranged from 2 to 7 mg/day in this individual. On day 1 of ethanol consumption and continuing for 4 days, aldosterone excretion rose dramatically. Values reached 30 mg/day, and blood-alcohol

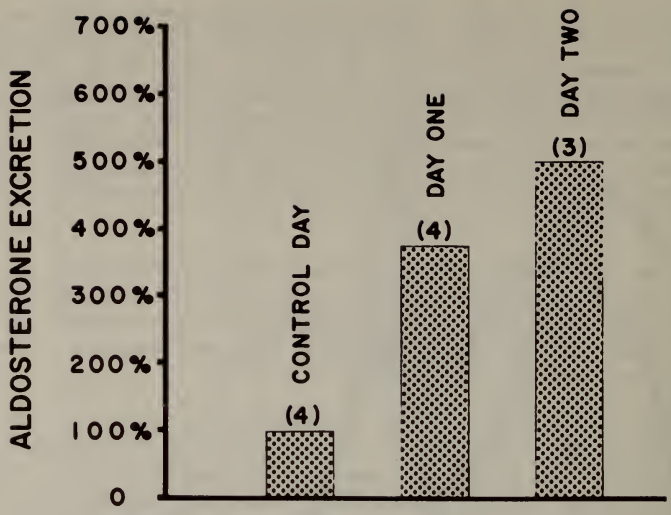
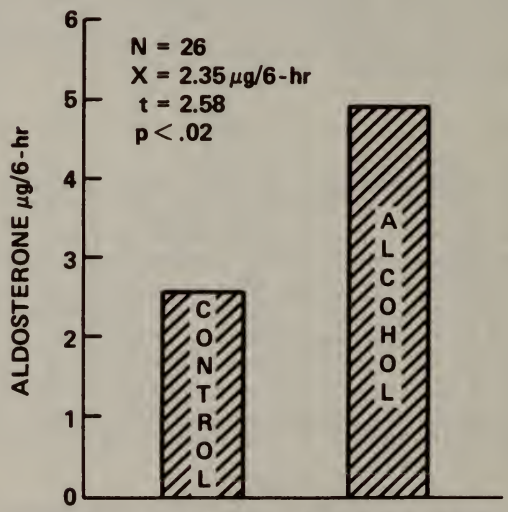


FIGURE 3.--Aldosterone excretory rates for four subjects during day 1 and day 2 of the ethanol ad lib period, relative to the last day of the control period



*Collections began 24 hrs, after onset of ethanol consumption in the group receiving ethanol.

FIGURE 4.--Average total aldosterone excretion per 6-hour period during control and 24 hours after ad lib consumption of alcohol (paired t-test analysis of the data)

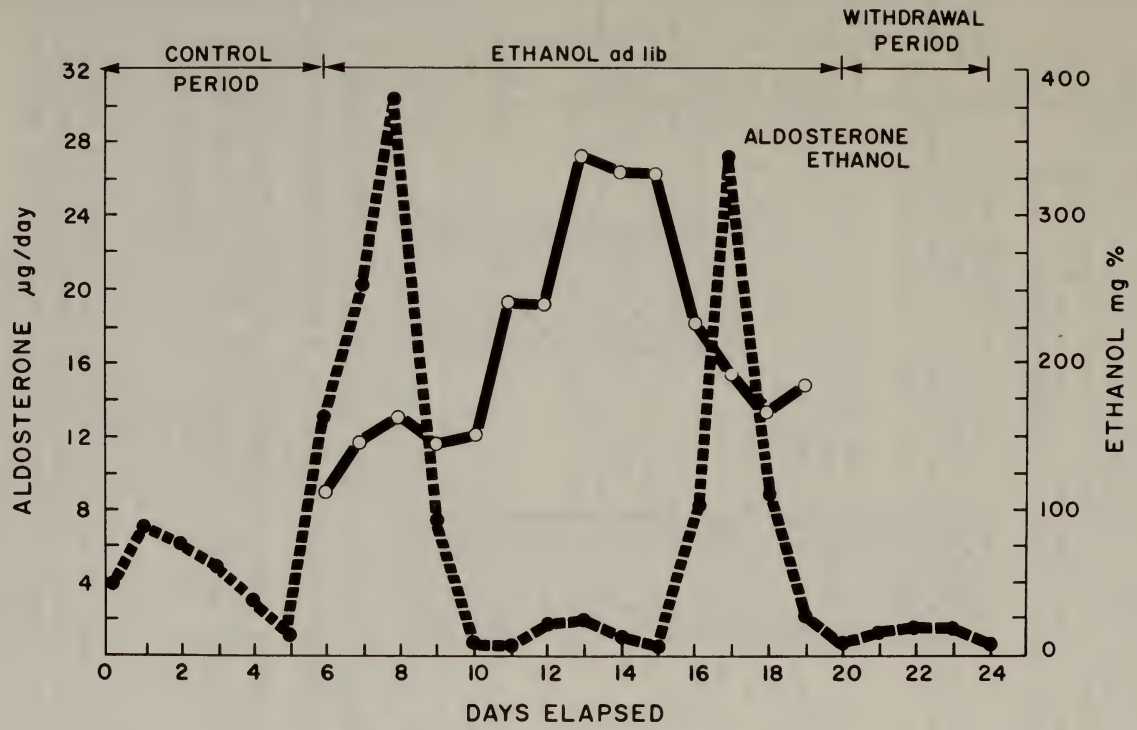


FIGURE 5.--Sequential daily urinary aldosterone excretion during control, ethanol ad lib, and withdrawal periods for a single subject

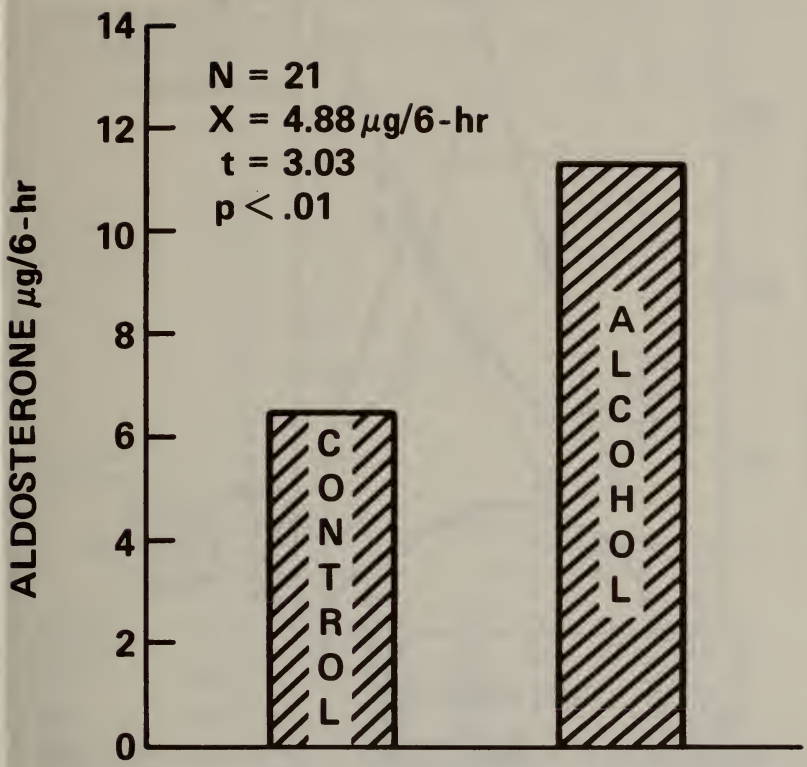
levels were 100 to 150 mg percent. Aldosterone excretion then fell to normal or subnormal levels while the blood-alcohol concentration increased to as high as 350 mg percent. Later, as the individual tapered his ethanol consumption in anticipation of abrupt withdrawal, aldosterone excretion increased again up to 28 mg/day. The blood-alcohol levels ranged from 150 to 200 mg percent. After withdrawal, aldosterone levels fell to normal or subnormal levels (Fabre et al. 1972b).

These results seem to corroborate the dose-response findings in the dog; high levels of aldosterone are associated with blood-alcohol levels of 100 to 200 mg percent, and low levels of aldosterone are associated with high blood-alcohol levels.

Using a tritiated tracer and compartmental analysis techniques, estimates of aldosterone secretion were obtained in 11 subjects. As before, control levels were obtained, and each subject served as his own control. Then 24 hours after ethanol ingestion began, repeat measurements were performed. In figure 6 control aldosterone secretory rates average 6.42 mg/6 hours. After receiving ethanol ad lib for 24 hours, the aldosterone secretory rates increased to an average of 11.3 mg/6 hours. For these subjects, analysis revealed a difference of 4.88 mg/6 hours, significant at $p = .01$ (Fabre et al. 1971). These results imply that the increase in aldosterone excretion is due to increased aldosterone secretion and not to other factors. The results confirm and extend the animal data.

In nonalcoholic subjects, the effect of alcohol on aldosterone secretion is somewhat different. Figure 7 depicts the results for five normal young men who received either orange juice or orange juice plus vodka on 2 consecutive days. For normal males, it is difficult to obtain blood-ethanol levels greater than 100 mg percent or to maintain these at that level for long. In this experiment, on the average, blood-ethanol levels just reached 100 mg percent and then only for 2 hours. Plasma aldosterone did not increase (220 percent of control, $p = .05$) until blood-ethanol levels began to fall and the subjects were having acute distress. Before the rise in plasma aldosterone, plasma renin rose to almost 400 percent of control ($p = .05$), and during the rise in plasma aldosterone levels, plasma cortisol levels also rose greatly ($p = .05$). These results suggest that the increase in plasma adolsterone is mediated by stress-induced factors, such as ACTH and the renin-angiotensin system. It is important to note that here we are measuring plasma aldosterone rather than aldosterone secretion or excretion. Obviously, plasma adolsterone levels are a result of many factors, most of which seem to be altered by alcohol (Farmer and Fabre 1974b).

The mechanism by which ethanol stimulates aldosterone secretion then became the focus of attention. Early studies had shown that ethanol had no effect in vitro on the biosynthesis of aldosterone by the zona glomerulosa of either human or canine



*Secretion values are calculated from compartmental analysis of 9 μC/tritiated aldosterone.

*Subjects receiving alcohol received tritiated aldosterone 24 hours after onset of alcohol consumption.

FIGURE 6.--Average total aldosterone secretion in μg/6 hours for subjects during control and ethanol ad lib periods (data analyzed by paired t-test)

adrenals (Fabre et al. 1969a). Presumably, then, the control factors for aldosterone--i.e., the renin-angiotensin system and ACTH--must be involved. The results of simultaneous measurement of all these parameters in a single abstaining alcoholic

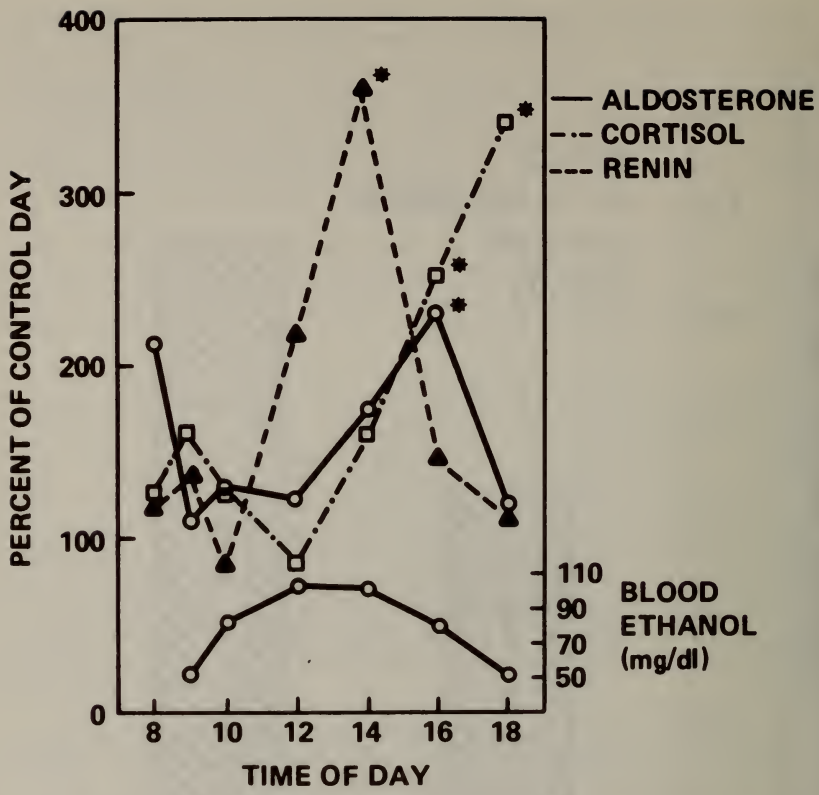


FIGURE 7.--Effect of acute ethanol consumption on plasma aldosterone, cortisol, and renin activity in normal young men who consumed vodka (expressed as percent of control day). Asterisks denote paired t-test significant differences from the control day values, $p = .05$.

subject who was administered ethanol ad lib are shown in figure 8 (Fabre et al. 1972b). The pattern of urinary aldosterone excretion is similar to that reported previously. There is an initial rise associated with the onset of drinking, and then these values fall to normal or subnormal levels. On withdrawal, the values again rise to very high levels. Blood-ethanol levels for this subject were high throughout the drinking phase, often above 300 mg percent. At the onset of drinking, both plasma renin activity and plasma cortisol activity increased. However, the increase in these two parameters lagged behind the increase in aldosterone excretion, suggesting they were not involved. The increase in aldosterone during withdrawal was preceded by increases in plasma renin and plasma cortisol, suggesting a role at this point.

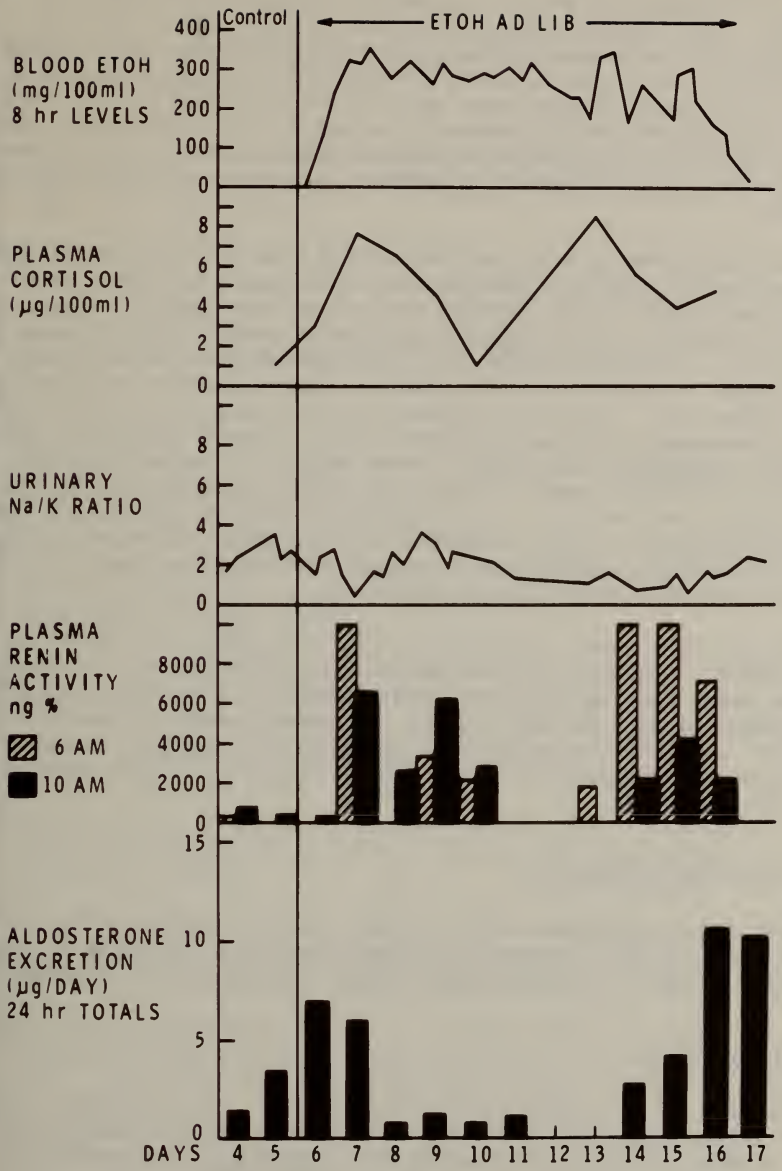


FIGURE 8--Parameters of aldosterone metabolism in a chronic alcoholic subject during control and ethanol ad lib period

DISCUSSION

The foregoing experiments in animals, abstaining alcoholic subjects, and normal subjects show that ethanol has a dramatic effect on aldosterone metabolism. Aldosterone secretion, urinary excretion, and plasma levels all reveal this effect. The effect is observed at blood-ethanol levels (100 to 300 mg percent) that are not uncommon in the clinical situation. It is, therefore, probable that ethanol-induced changes in aldosterone metabolism are important in intoxication, withdrawal, and chronic alcohol consumption.

The mechanism by which ethanol alters aldosterone secretion has been only partially elucidated. At the onset of drinking, aldosterone levels increase before cortisol or renin levels, suggesting that these latter mechanisms play a lesser role in this phenomenon. However, ethanol inhibition of vasopressin, with diuresis and changes in hemodynamic parameters, undoubtedly is responsible in some way. Acute hypovolemia is one of the most potent stimuli to aldosterone secretion (Bartter 1956). During prolonged alcoholic drinking, vasopressin levels return to normal (Eggleton 1942). As seen here, aldosterone levels then return to normal or subnormal levels. It is likely that these two parameters are interrelated. A direct inhibition of aldosterone secretion by high levels of blood ethanol must also be considered, as shown in the animal studies (Fabre et al. 1972a).

During withdrawal, aldosterone levels rise again. The data here, however, suggest that the stress of withdrawal results in increased renin activity and ACTH activity, which are responsible at least in part for this rise in aldosterone levels. Thus, puffiness, swollen feet, and weight gain after a night on the town are probably caused by the effect of alcohol on aldosterone metabolism.

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Pseudo-Cushing Syndrome and Alcohol Abuse

David H. Van Thiel, Judith S. Gavaler, and C. F. Cobb

Cushing's syndrome is the generic name for cases of advanced glucocorticoid overproduction, characterized by clinical signs that include central obesity, cutaneous striae, osteoporosis, weakness, hypertension, diabetes, plethora, and hirsutism (Baxter and Tyrrell 1981; Liddle 1981). True Cushing's disease, in contrast, is a disorder resulting from oversecretion of pituitary ACTH (corticotropin) and is therefore one of the ACTH-dependent types of Cushing's syndrome. (See table 1.)

The endocrine abnormalities that occur in Cushing's disease include:

(1) Hypersecretion of ACTH and cortisol;

(2) Absent circadian rhythm for ACTH and cortisol;

(3) Abnormal ACTH and cortisol responses to stress;

(4) Abnormal negative feedback regulation of ACTH secretion by glucocorticoids (reduced ACTH suppressibility with dexamethasone and hyperresponsiveness to inhibition of cortisol synthesis with metapyrone); and

(5) Subnormal responses of growth hormone (GH), thyrotropin (TSH), and gonadotropins [FSH (follicle-stimulating hormone) and LH (luteinizing hormone)] to various stimuli (not universal, however).

Because Cushing's disease is ACTH dependent, it usually is treated either directly by hypophysectomy or hypophyseal ablation using some form of irradiation. In unusual situations, however, it is treated by bilateral adrenalectomy.

Pseudo-Cushing's syndrome, like Cushing's disease, is characterized by elevated basal cortisol levels, loss of the normal diurnal variation, and failure of overnight dexamethasone suppression (table 1). Two forms of pseudo-Cushing's syndrome are generally recognized: one that is caused by chronic alcohol abuse

TABLE 1.--Findings in Cushing's syndrome and pseudo-Cushing's syndrome

Cushing's syndrome and Cushing's disease	Pseudo-Cushing's syndrome	
	Alcoholism	Depression
Elevated basal ACTH and cortisol levels	Elevated basal cortisol levels; ACTH normal or reduced	Elevated basal ACTH and cortisol levels
Abnormal diurnal variation	Abnormal diurnal variation	Abnormal diurnal variation
Abnormal dexamethasone suppression	Abnormal dexamethasone suppression	Abnormal diurnal variation
Abnormal ACTH and cortisol responses to stress	Abnormal ACTH and coritsol responses to stress and insulin-induced hypoglycemia	Normal cortisol response to insulin-induced hypoglycemia
Abnormal (reduced) basal and stimulated GH, TSH, FSH, and LH responses to various stimuli	Increased basal GH and abnormal TRH-stimulated GH responses; reduced LH responses to LRF; increased basal TSH but abnormal responses to TRH	Normal basal and stimulated GH levels; normal LH response to LRF; abnormal TSH response to TRH

(alcoholism)--the subject of this chapter--and a second form that is caused by depression (Baxter and Tyrrell 1981, pp. 465-468). These two conditions differ from true Cushing's disease in that: (1) in depression the normal response to insulin-induced hypoglycemia remains intact, and (2) in alcoholism the basal and TRH-stimulated GH responses are increased; basal TSH levels are increased slightly, the TSH response to TRH (thyrotropin-releasing hormone) is abnormally low, and the luteinizing hormone (LH) responses to LRF (luteinizing releasing factor) is reduced (Van Thiel et al. 1978a,b, 1979). Thus, the two forms of pseudo-Cushing's syndrome can be differentiated easily from true Cushing's syndrome if: (1) the nature of the underlying condition, either alcoholism or depression, is sought and determined; (2) the response to insulin-induced hypoglycemia is evaluated--it is abnormal in true Cushing's syndrome and in alcoholism but is intact in depression; and (3) basal and stimulated levels of the other pituitary hormones are examined--they are reduced in the true Cushing's syndrome and are usually elevated in alcoholism.

Although the pseudo-Cushing's syndrome that occurs with alcoholism has been described only relatively recently, it should not have been unexpected. Prior experimental studies with animals had clearly predicted its ultimate recognition in humans (table 2). Specifically, ethanol administration to rodents had been

TABLE 2.--Effects of ethanol on the hypothalamic-pituitary-adrenal axis of experimental animals

1951-52	Smith, Forbes, and Duncan report that ethanol administration reduces the rodent adrenal gland content of ascorbate and cholesterol.
1966	Ellis reports: (1) dose-response increase in corticosterone in response to ethanol in mice; (2) failure of adaptation of the response; and (3) hypophysectomy or morphine plus phenobarbital block the response.
1971	Noble et al. report that: (1) the corticosterone response to ethanol occurs in alcohol-adapted as well as alcohol-naive animals and exceeds response to saline injection; and (2) corticosterone clearance and response to exogenous ACTH are identical in alcohol-naive and alcohol-adapted mice.
1975	Kakihana and Moore report that mice given alcohol chronically have larger adrenal glands than do controls.
1981	Cobb et al. report that both ethanol and acetaldehyde, at concentrations achievable in alcohol-ingesting humans, stimulate the isolated perfused rat adrenal to hypersecrete corticosterone.

shown to deplete the adrenal gland content of ascorbate and cholesterol and to be associated with increased plasma glucocorticoid levels as well as adrenal hypertropy (Smith 1951; Forbes and Duncan 1951, 1952, 1953; Kakihana and Moore 1975; Noble et al. 1971). Similarly, early experimental studies in humans should have predicted its occurrence and early recognition (table 3). These studies include the finding that ethanol administration to humans is followed by an increase in cortisol levels and that the response is related to both dose and time but, most importantly, that humans appear to be more sensitive to ethanol in terms of glucocorticoid responsiveness than are rodents (Mendelson and Stein 1966; Frazekas 1966; Jenkins and Connolly 1968; Bellett et al. 1970; Mendelson et al. 1971; Merry and Marks 1972; Stokes 1973).

The factors that probably delayed an earlier recognition of alcoholic pseudo-Cushing's syndrome were the independent observations that alcoholics had reduced cortisol responses to surgical stress, insulin-induced hypoglycemia, and exogenous ACTH than did controls (table 3) (Margraf et al. 1967). Compounding these findings in alcoholics as a group was the additional information that several cases of adrenal insufficiency and rare cases of selected ACTH deficiency were described in alcoholics as early as 1949 and through the mid-1960s (Tintera and Lovell 1949; Smith 1950; Freinkel et al. 1963; Woeber and Arky 1965). Thus, clinicians began to expect adrenal insufficiency rather than hypersecretion in chronic alcohol abusers.

TABLE 3.--Effects of alcohol on the hypothalamic-pituitary-adrenal axis in humans

1966	Mendelson and Stein report that ethanol administration to humans increases cortisol levels and note that the response is related to both dose and time.
1967	Margraf et al. report that alcoholics have reduced cortisol responses to exogenous ACTH and to surgical stress as compared with controls.
1968	Jenkins and Connolly confirm work of Mendelson and Stein (1966).
1970	Bellet et al. reconfirm the findings of Mendelson and Stein (1966) and Jenkins and Connolly (1968) but also note that the GH responses are exaggerated as well.
1971	Mendelson et al. report again that ethanol increases cortisol levels in humans but add that the adrenocortical response of humans to ethanol is more sensitive than that of the rodent.
1972	Merry and Marks report that alcoholics have inadequate cortisol responses to insulin-induced hypoglycemia as compared with controls.

TABLE 4.--Published cases of alcohol-induced pseudo-Cushing's syndrome

1976	(November)	Smals et al.: two men and one woman, all three have classic Cushingoid appearance, and all three failed to suppress with dexamethasone (2 mg); all three returned to normal during 3 weeks of alcohol abstinence in the hospital.
1976	(December)	Paton: five men and three women, all with classic features of Cushing's syndrome; all had alcoholic hepatitis/cirrhosis on liver biopsy.
1977	(April)	Rees et al.: two men and two women; all four had elevated cortisol levels; two failed to suppress in response to dexamethasone (2 mg); three had reduced or undetectable ACTH levels; all four returned to normal in hospital during alcohol abstinence.
1977	(May)	Frajria and Angeli: two men failed to suppress with dexamethasone (1 mg); had reduced response to insulin-induced hypogylcemia; normal response to exogenous ACTH and lysine vasopressin; both had cirrhosis.
1977	(December)	Smals and Kloppenborg (1977): describe three additional patients to 1976 study by Smals et al.; all six had lost the normal diurnal variation; three failed to suppress with dexamethasone (2 mg).
1978	(December)	Binkiewiez et al.: pseudo-Cushing's syndrome in an infant; mother was a known alcoholic with blood-alcohol level of 100 mg/dl in her breast milk.

Despite such misdirected expectations, alcoholic pseudo-Cushing's syndrome was first identified and quickly confirmed in 1976 (table 4) (Smals et al. 1976; Paton 1976). Additional cases were recognized and added in 1977 and 1978, until the syndrome became so well known that new cases no longer merited publication (Rees et al. 1977; Frajria and Angeli 1977; Smals and Kloppenborg 1977; Smals et al. 1977; Binkiewiez et al. 1978).

There are differences between the two syndromes, however. In contrast to true Cushing's syndrome, pseudo-Cushing's syndrome of alcohol abuse occurs more frequently in males than in females and in a slightly older population; it is associated with chronic

alcoholism; and perhaps even more importantly, liver function tests and liver histopathology are abnormal and are consistent with chronic alcohol abuse (table 5) (Smals et al. 1976, 1977; Paton 1976; Rees et al. 1977; Frajria and Angeli 1977; Smals and Kloppenborg 1977). A final criterion for distinguishing the two syndromes is ACTH level. These levels are invariably increased in true Cushing's syndrome and are normal or reduced in alcoholic pseudo-Cushing's syndrome (Baxter and Tyrrell 1981, p. 477; Liddle 1981; Rees et al. 1977). In perplexing cases, assay of the other pituitary hormones will help to confirm the diagnosis. With true Cushing's syndrome, the plasma level of all of the other hormones of the anterior pituitary are normal or reduced, but in alcoholic pseudo-Cushing's syndrome, they tend to be either high normal or increased (Van Thiel et al. 1978a,b, 1979).

The results reported to date concerning hormone levels and their response times to various stimuli leave one somewhat in doubt as to the specific pathogenesis of alcohol-induced pseudo-Cushing's syndrome. Data for animals suggest that ethanol administration results in the initiation of a centrally perceived stress, which is followed by ACTH release that secondarily accounts for the increase in plasma levels of adrenocortical hormones (Ellis 1966). In contrast, data for humans suggest that alcoholics as a group are more likely to have ACTH deficiencies (Tintera and Lovell 1949; Smith 1950; Freinkel et al. 1963; Woeber and Arky 1965) and reduced adrenocortical responses to various stresses, such as surgery and insulin-induced hypoglycemia (Margraf et al. 1967).

How then does the pseudo-Cushing's syndrome occur? Is the stimulus ethanol or acetaldehyde, or could it be something else? Answers seem to be forthcoming, but slowly. Specifically, both ethanol and acetaldehyde have been shown to stimulate the isolated perfused rat adrenal to secrete corticosterone, with the latter agent being about 1,000-fold more active, based on the molar dose of drug required to produce a response (Ellis 1966). Data from isolated Leydig cells, which are not too different from adrenocortical cells, have shown that ethanol exposure results in the secretion and release of cyclic AMP (adenosine monophosphate) from such cells (Rajum et al. 1982). Because cyclic AMP is the established second message following ACTH binding to adrenal cortical cells, it is possible that ethanol and possibly acetaldehyde stimulate adrenocortical synthesis and secretion of corticosteroids even in the absence of ACTH by activating adenyl cyclase. Consistent with such a hypothesis is the additional finding that acetaldehyde stimulates cyclic c-AMP generation and release by isolated adipose tissue cells.

Clearly, much remains to be accomplished if our understanding of the pathogenesis of the pseudo-Cushing's syndrome due to chronic alcoholism is to be advanced. However, because the syndrome is recognized in humans and because we have considerable experimental data to support such a condition in experimental animals, the next advance in our understanding will probably occur at the molecular level.

TABLE 5.--True Cushing's syndrome and alcoholic pseudo-Cushing's syndrome

	True Cushing's syndrome	Pseudo-Cushing's syndrome
Sex	Females > males 3:1 to 10:1	Males > females 2:1
Age	20 to 40	30 to 60
Liver function	Normal	Abnormal with increased alkaline phosphatase, α-GTP (guanosine triphosphate) bilirubin, and transaminases
Liver biopsy	Normal to slightly fatty	Alcohol hepatitis/cirrhosis
History of alcohol abuse	Rare	Essential
ACTH levels	Increased	Reduced or nondetectable
GH levels	Increased	Increased
TSH levels	Normal or reduced	Slight increase
LH levels	Normal or reduced	Normal or increased

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Alcohol-Induced Alterations in Gastrointestinal Hormone Secretions and Actions

Linda L. Shanbour

INTRODUCTION

The influence of alcohol on gastrointestinal hormone secretions and actions depends upon numerous factors: (1) previous exposure to ethanol or other damaging agents, (2) the concentration of the alcohol, (3) duration of alcohol exposure, (4) route of administration of alcohol, and (5) nutritional status of the subject exposed to alcohol. In addition, there is considerable interaction between the gastrointestinal hormones (figure 1). Gastrin is released from the antrum of the stomach and circulates via the blood to the fundic portion of the stomach where it stimulates acid secretion. Hormones released from the small intestine that

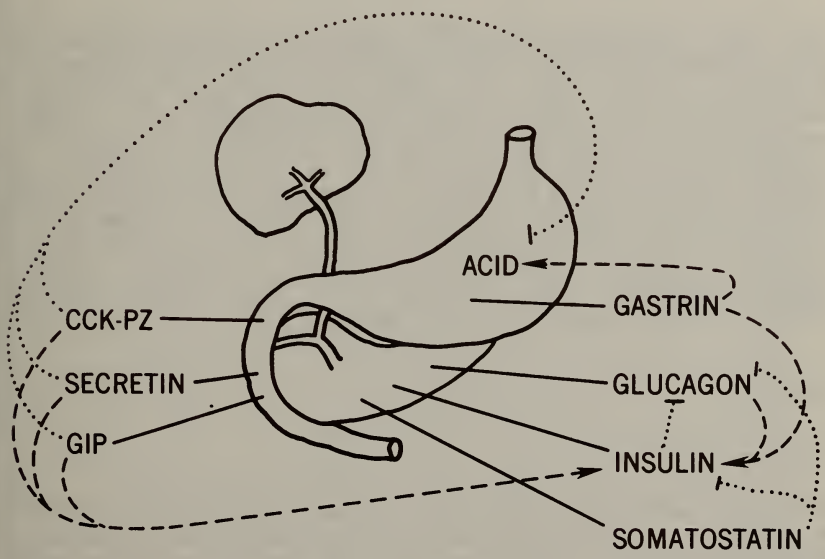

FIGURE 1.--Interactions of gastrointestinal hormones

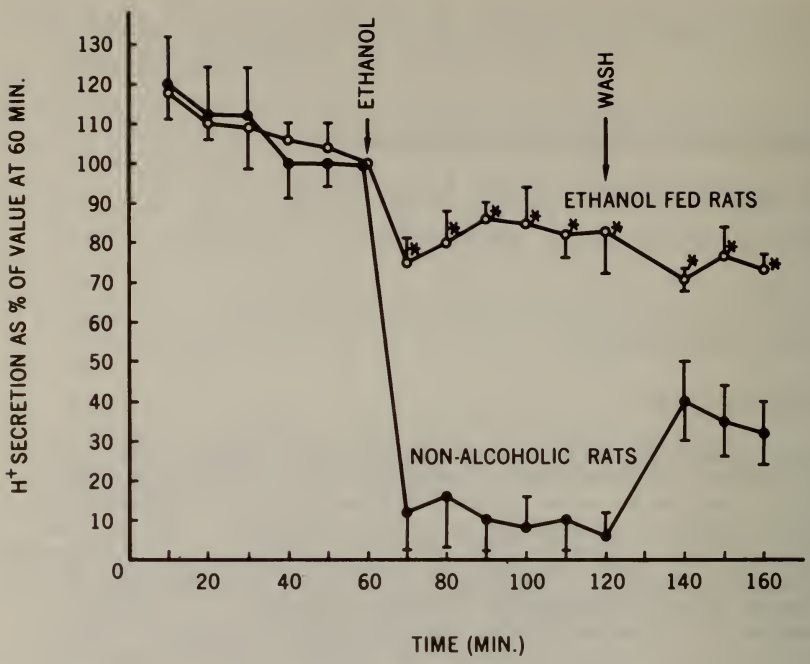

FIGURE 2.--Effects of mucosal ethanol (6 percent) on basal secretion in isolated rat gastric mucosa

have been implicated in alcohol's actions are cholecystokinin-pancreozymin (CCK-PZ), which stimulates pancreatic enzyme secretion, secretin, which stimulates pancreatic water and bicarbonate output, and gastric inhibitory polypeptide (GIP). These hormones inhibit gastric acid secretion and stimulate insulin release from the pancreas (Unger et al. 1967). Glucagon, released primarily from the α cells of the islets, and gastrin also stimulate insulin secretion (Unger et al. 1967). Somatostatin, released from the delta cells of the islets, inhibits insulin and glucagon secretion (Efendic 1977). Insulin and secretin inhibit glucagon secretion (Unger and Orci 1976). The action of alcohol on each of these hormones is still debated.

GASTRIN

ACID SECRETION

The direct action of alcohol on the stomach under acute conditions is inhibition of acid secretion in a variety of species, including rat (Shanbour et al. 1973; Puurunen and Karppanen 1975), rabbit (Fromm and Robertson 1976; Mierson et al. 1980), dog (Sernka

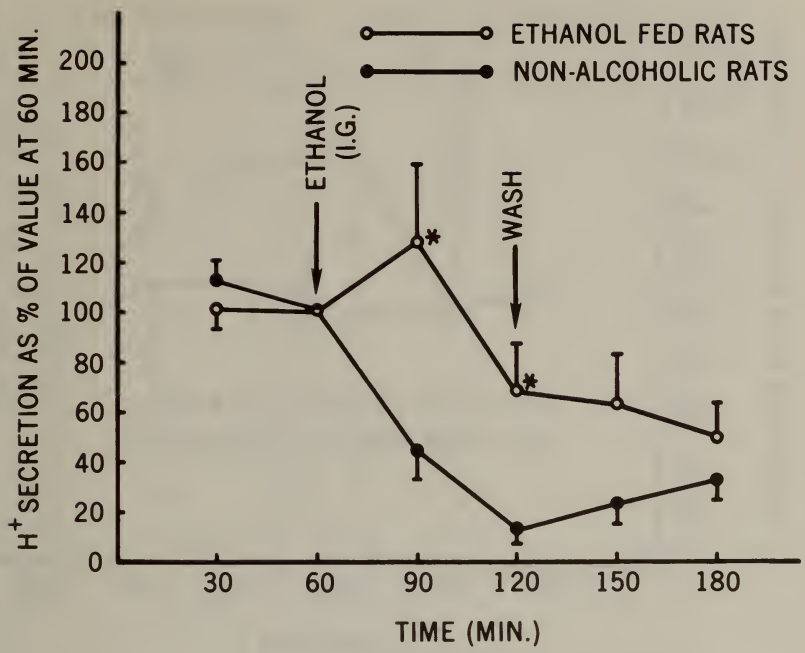

FIGURE 3.--Effects of intragastric ethanol (20 percent, 2 ml) on basal secretion of rat stomach in vivo

et al. 1974; Kuo and Shanbour 1980), and human (Cooke 1972). This inhibition of acid secretion has been demonstrated both in vivo (Shanbour et al. 1973; Cooke 1972; Puurunen and Karppanen 1975; Sernka et al. 1974) and in vitro (Fromm and Robertson 1976; Kuo and Shanbour 1980; Mierson et al. 1980). Alcohol also inhibits other energy-requiring active transport processes in the stomach (Kuo et al. 1974; Shanbour and Kuo 1979; Shanbour 1980). However, chronic exposure to alcohol will modify this response. In recent studies from our laboratory (Kuo and Shanbour in press), rats were fed 20 percent ethanol, 4 g/kg, daily by gastric intubation for 28 days. In one series of studies (figure 2) gastric mucosae, with muscle layers stripped off, from ethanol-fed and nonalcoholic (saline control) rats were mounted in vitro chambers, and acid secretion was measured. Ethanol, 20 percent to the mucosal side, decreased acid secretion in both groups and had a much more pronounced effect in the naive, nonalcoholic rats. In a second series (figure 3), acid secretion was measured in the intact stomach in response to 20 percent ethanol administration intragastrically (IG). At 30 minutes after ethanol treatment, which corresponded to a peak blood-ethanol concentration of 120 mg percent, there was an increase in acid secretion in the ethanol-fed rats; otherwise, the ethanol inhibited

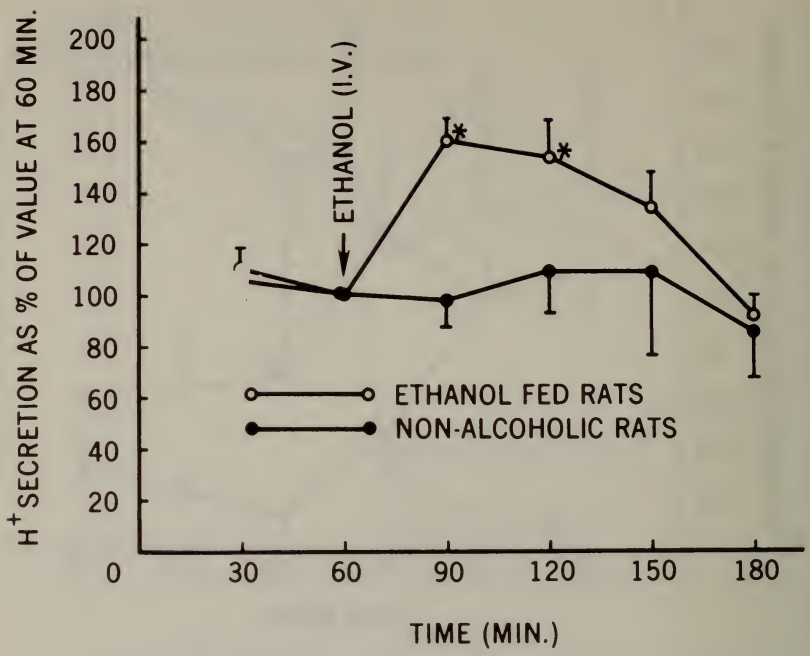

FIGURE 4.--Effects of intravenous ethanol (0.8 g/kg) on spontaneous acid secretion of rat stomach in vivo

acid secretion. In a third series of experiments (figure 4), 20 percent ethanol, 0.8 g/kg, was infused intravenously (IV) for 5 minutes. There was essentially no change in acid secretion in the naive, nonalcoholic rats. However, the IV administered ethanol increased acid secretion in the chronically ethanol-fed rats.

SERUM GASTRIN

Studies have been designed to determine whether ethanol releases antral gastrin and if so, whether the amount released is sufficient to stimulate fundic acid secretion (Bo-Linn and Shanbour 1977). In acute dog studies, a denervated antral pouch with blood supply maintained intact was constructed for instillation of ethanol, and the fundic portion of the stomach with blood supply maintained intact was mounted in a chamber for measurement of acid output and potential difference. Only at the 20 percent concentration did ethanol produce a measurable increase in serum gastrin, but this was not sufficient to stimulate acid secretion from the fundic segment or to alter the potential difference (figure 5). The known gastrin-releasing agent, glycine, increased serum gastrin

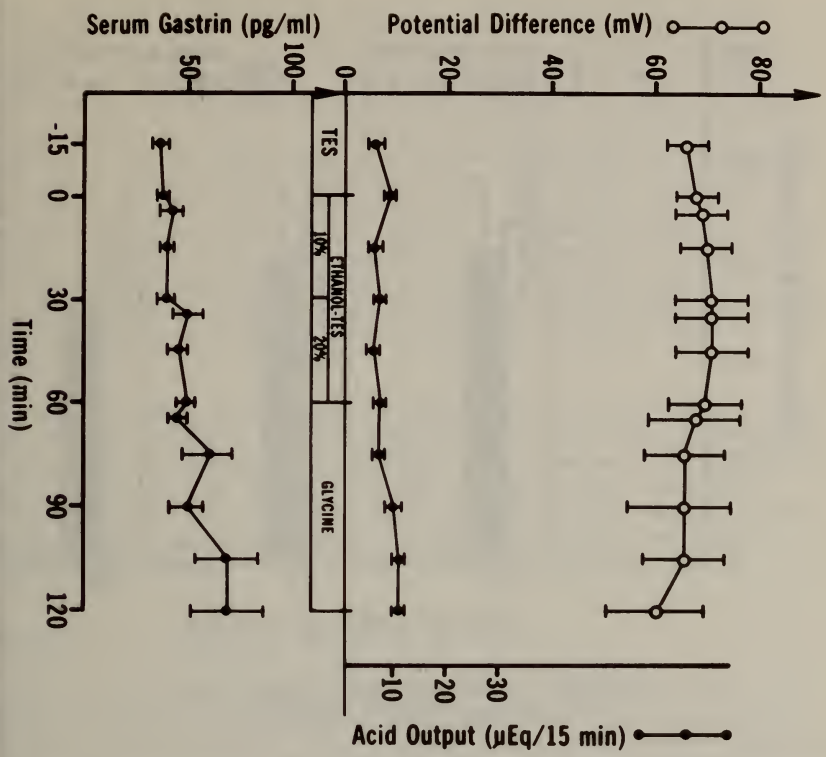

FIGURE 5.--Potential difference (mV), acid output (µEq/15 minutes), and serum gastrin (pg/ml) with time during antral perfusion with TES (N-tris[hydroxymethyl]methyl-2-aminoethane sulfonic acid) buffer, ethanol in TES buffer (10 and 20 percent), and glycine at pH 7.4

with a corresponding tendency toward an increase in acid secretion and decrease in potential difference in the fundic portion of the stomach.

The chronic effects of ethanol on serum gastrin were determined in the above-mentioned studies (Kuo and Shanbour 1982) on chronic acid secretion in rats. During the 28 days of chronic ethanol feeding there were no significant differences in serum gastrin with time when the ethanol-fed rats were compared with the naive, nonalcoholic rats (figure 6). We also found that atropine, a cholinergic blocker of acid secretion, did not affect the increase in acid secretion produced by IV infusion of ethanol. However, cimetidine, the H_2-receptor blocking agent, abolished the increase in acid secretion. These studies suggest that histamine may be the mediator of the increased acid secretion with chronic ethanol feeding.

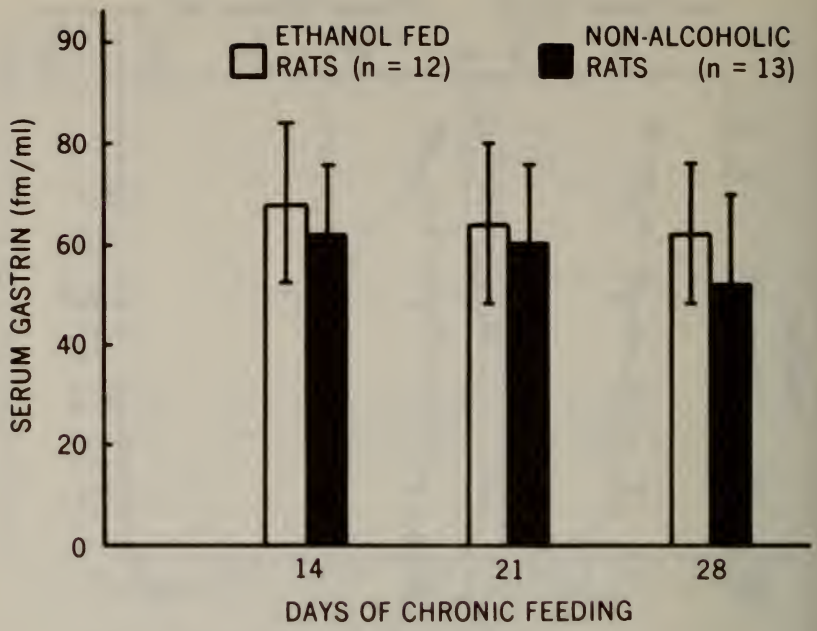

FIGURE 6.--Serum gastrin levels in rats during chronic feeding

Table 1 summarizes the effect of alcohol on gastrin release. Bioassay for gastrin, which consisted of measurements of acid secretion from Heidenhain pouches, gastric fistulas, or fundic chamber, have failed to show any changes in acid secretion in response to antral administration of ethanol. The only consistent reports of increases in serum gastrin in response to ethanol as measured by radioimmunoassay (RIA) are from the laboratories of Thompson (Becker et al. 1974; Llanos and Thompson 1977) and Sarles (Treffot et al. 1975, 1980). Becker et al. (1974) reported an increase in gastrin in the antral vein when ethanol was instilled into the antral pouch or given IV in dogs but did not report values for peripheral gastrin or acid secretion. They showed an increase in gastrin from the peripheral vein in response to oral and IV administered ethanol in humans, but the effects were not dramatic except for the 50 percent oral administration, and they did not measure acid secretion in the human subjects. Also, prior exposure to alcohol in these human subjects could not be clearly determined. Llanos and Thompson (1977) reported an increase in RIA gastrin when ethanol was given orally to humans or through a gastric fistula in dogs but found no effect in humans, dogs, or cats when ethanol was placed in the duodenum. Treffot et al. (1975) reported that 50 percent ethanol given acutely or chronically through gastric fistulas of dogs increased

serum gastrin, and a much greater response was observed in the 30-month chronically ethanol-fed dogs. They atttributed the greater response in these animals to hyperplasia of the gastrin cells. Treffot et al. (1980) in studies on human subjects who ingested 40 percent ethanol orally showed that gastrin levels are increased in chronic calcifying pancreatitis and chronic alcoholics but not in normal control subjects or subjects with alcoholic cirrhosis. Since gastric hypoacidity has been shown to be associated with chronic pancreatitis (Kravetz and Spiro 1965) and chronic alcoholism (Chey et al. 1968), the elevated gastrin release could have been due to a decrease in the feedback inhibition by a low pH (Gedde-Dahl 1974; Trudeau and McGuigan 1971). The lack of change in gastrin levels in subjects with alcoholic cirrhosis was attributed to possible gastritis with a decreased mass of G cells.

In summary, the direct effect of ethanol on the stomach is inhibition of acid secretion. High concentrations of ethanol administered orally or IG are required to increase plasma serum gastrin, which may or may not be associated with increased acid secretion. Chronic studies with IG or IV administered ethanol suggest that histamine may be the mediator in the altered acid secretory response to ethanol.

SECRETIN

Secretin is released from the duodenum in response to acid. In human subjects, Straus et al. (1975) found an increase in RIA secretin with no change in RIA gastrin when ethanol was ingested. They attributed these findings to a direct effect of ethanol on the duodenal mucosa. However, they did not measure acid secretion. Llanos and Thompson (1977) were able to obtain an increase in secretin, as well as gastrin, with oral ingestion of ethanol in humans but not with duodenal installation of ethanol in humans, dogs, and cats. They also failed to measure acid secretion in their studies. In contrast, in chronic studies on dogs that received ethanol orally for 3 years, Bretholz et al. (1978) found a decrease in secretin levels. Thus, ethanol probably does not release secretin directly from the duodenal mucosa, or any direct release is too small to detect. However, correlation studies of acid secretion and hormone levels are necessary to clarify this issue.

CHOLECYSTOKININ-PANCREOZYMAN AND GASTRIC INHIBITORY POLYPEPTIDE

Few studies have been done on the effects of ethanol on the release of CCK-PZ and GIP from intestinal mucosa. Planche et al. (1977) found that CCK-PZ blood levels in response to an intraduodenal oleic acid injection are not modified by chronic alcohol consumption in dogs that were fed ethanol for 3 years. Wright and Marks (1980) found an increase in plasma GIP concentration in human subjects who consumed an alcohol and sucrose tonic,

TABLE 1.--Effect of alcohol on gastrin, secretin, CCK-PZ, and GIP

Authors	Species	Method	Dose or concentration	Gastrin		Secretin	CCK-PZ	GIP
				Bioassay	RIA	RIA	RIA	RIA
Cooke and Grossman (1968)	Dog	Antral pouch	5 to 10 percent					
		Heidenhain pouch		↔				
		Gastric fistula		↔				
Cooke and Birchall (1969)	Human	IG	0.79 to 5.58 g percent	↔				
Cooke (1972)	Human	Oral	8-16 g percent	↓				
Becker et al. (1974)	Dog	Antral pouch	10 percent		↑(antral vein)			
		IV	10 percent		↑(antral vein)			
		Oral	18-50 percent		↑(peripheral vein)			
		IV	7 percent		↑(peripheral vein)			
Raptis et al. (1974)	Human	Oral	19 percent		↔			
Treffot et al. (1975)	Dog	Gastric fistula:						
		Acute	50 percent		↑			
		Chronic	50 percent		↑			
Bo-Linn and Shanbour (1977)	Dog	Antral pouch	10 to 20 percent					
		Fundic chamber		↔	sl.↑			

Treffot et al. (1980)	Human:	Oral	40 percent				
	Normal			↔			
	Pancreatitis			↑			
	Alcoholic			↑			
	Cirrhosis			↔			
Kuo and Shanbour (in press)	Rat, chronic	IG	20 percent	↔			
		IV	20 percent (0.8 g/kg)	↔			
Straus et al. (1975)	Human	Oral	0.4 g/kg	↔	↑		
Llanos and Thompson (1977)	Human	Oral +	0.4 g/kg	↑	↑		
		Duodenum	0.4 g/kg	↔	↔		
	Dog	Gastric +	45 percent	↑	↔		
		Pancreatic fistulas	45 percent	↔	↔		
	Cat	Pancreatic fistula	45 percent	↔	↔		
Bretholz et al. (1978)	Dog, chronic	Oral	2 g/kg/day		↓		
Planche et al. (1977)	Dog, chronic	Oral	2 g/kg/day			↔	
Wright and Marks (1980)	Human	Oral	59 g				↑

TABLE 2.--Effect of alcohol on insulin and glucagon

Authors	Species	Method	Dose or concentration	Insulin		Glucagon	
				Fasted (hours)	Fed	Fasted	Fed
Singh and Patel (1976)	Rat	Intact	0.4 g/kg, IG	(18) ↔	(Glucose, IG) ↓		
Jauhonen (1978)	Rat	Intact	5 g/kg, IG	(24) ↔		↑	
Potter et al. (1980)	Rat	Intact	150 to 750 mg/100 g, IG	(18) ↓		↑	
Shah et al. (1977)	Rat	Intact	25 percent, IG	(12) ↓		↑	
Potter and Morris (1980)	Rabbit	Intact	20 percent, IG	(18) ↓		↑	
Tiengo et al. (1974)	Pig	Intact	6 mg/min/kg, IV	(18) ↓		↑	
Tiengo et al. (1979)	Pig	Intact	6 mg/min/kg, IV	(14) ↓	(Glucose, IV) ↔	↑	↔
Kuhl et al. (1976)	Pig	Intact	5 percent, IV		(Glucose, IV) ↑		
Bleicher et al. (1964)	Dog	Intact	15 percent, IV	(24) ↔			
Kuhl and Andersen (1974)	Human	Intact	5 percent, IV	(10) ↔	(Glucose, IV) ↑		
Palmer and Ensinck (1975)	Human	Intact	15 percent, IV	(56) ↓		↑	
Bagdale et al. (1972)	Human	Intact	236 mg/min, IV	(72) ↓			

Metz et al. (1969)	Human	Intact	12 percent, oral and IV		(Dextrose, IV)↑		
Singh et al. (1976)	Human	Intact	5 percent, IV		(Glucose, IV)↑		
McMonagle and Felig (1975)	Normal and diabetic human	Intact	30 percent, oral		(Glucose, oral)↑		
Turner et al. (1973)	Normal, obese, and diabetic human	Intact	15 percent, IV	(36)↓			
Nikkila and Taskinen (1975)	Normal, obese, and diabetic human	Intact	20 percent, oral and 100 mg/min, IV		(Glucose, IV and oral)↑		
Bagdale et al. (1972)	Thin and obese human	Intact	236 mg/min, IV	(72)↓			
Joffe et al. (1977)	Alcoholic human	Intact	None	↔		↑	
Tiengo et al. (1981)	Rat	Isolated perfused	10 to 30m<u>M</u>	(24)↓		↔	
Samols and Stagner (1980)	Dog	Isolated perfused	16 to 252m<u>M</u>		(Glucose 200 mg percent)	↓	↓
Patel and Singh (1979)	Rat	Isolated islet incubation	67m<u>M</u>		(Glucose 16.7m<u>M</u>)↓		
Mao et al. (1981)	Rat	Isolated islet perfusion	20 to 160 mg percent		(Glucose 40 mg percent)↑		

but there was no change after alcohol and fructose tonic. Therefore, the increase in plasma GIP was probably due to the sucrose. More studies are necessary to fully clarify the influence of ethanol on these intestinal hormones.

INSULIN

The role of ethanol in altering carbohydrate metabolism, particularly in inducing hypoglycemic conditions, has been debated by many investigators. Table 2 summarizes the various methods (intact pancreas, isolated perfused pancreas, isolated islet incubation, and isolated islet perfusion), species studied (rat, rabbit, pig, dog, normal, thin, obese, diabetic, and alcoholic humans), concentrations, and routes of ethanol administration on fasted and fed insulin- and glucagon-blood concentrations. Ethanol either has no effect or decreases insulin levels in the fasted or basal secretory state (some studies are questionable as to the fasting state because of the short time interval). The decrease in insulin levels in the fasted state is usually attributed to inhibition of gluconeogenesis in the liver by ethanol (Krebs et al. 1969). An ethanol load administered IV or orally either has no effect or potentiates a glucose-mediated increase in plasma insulin levels in the intact animal. One exception is the study by Singh and Patel (1976), in which they gave ethanol IG to rats and found a decrease in insulin levels in response to an intragastric load of glucose.

To determine whether ethanol has a direct effect on pancreatic islets, Mao et al. (1981) used an isolated islet perfusion preparation in which minute-to-minute changes in insulin secretion could be measured in response to a continuous infusion of ethanol. Figure 7 illustrates that ethanol increases insulin secretion in a dose-dependent manner (20 to 160 mg percent) when there is a background of 40 mg percent glucose present. The ethanol effect was not altered by the alcohol dehydrogenase inhibitor, pyrazole; thus, ethanol directly affects the islets. The activity of alcohol dehydrogenase (ADH) is also quite low as compared with most other tissues (Guynn et al. 1982). The ethanol did not damage the islets because preinfusion of ethanol, followed by a wash, did not alter the islet respone to glucose. Table 3 shows data indicating that ethanol acts on β receptors of the β cells to stimulate insulin secretion. Epinephrine, the β-receptor blocking agent, propranolol, or the α-receptor blocking agent, phentolamine, failed to alter insulin secretion when administered alone. However, when ethanol and epinephrine or ethanol and propranolol were administered together, the insulin secretion was depressed as compared with ethanol alone. The combination of ethanol and phentolamine induced insulin secretion that was not significantly different from that induced by ethanol alone. Epinephrine is a nonspecific agonist of α and β receptors, and since α receptors predominate in the islets, an inhibitory effect of ethanol is observed with epinephrine. Phentolamine did not affect the insulin response to ethanol, thus showing that the α receptors

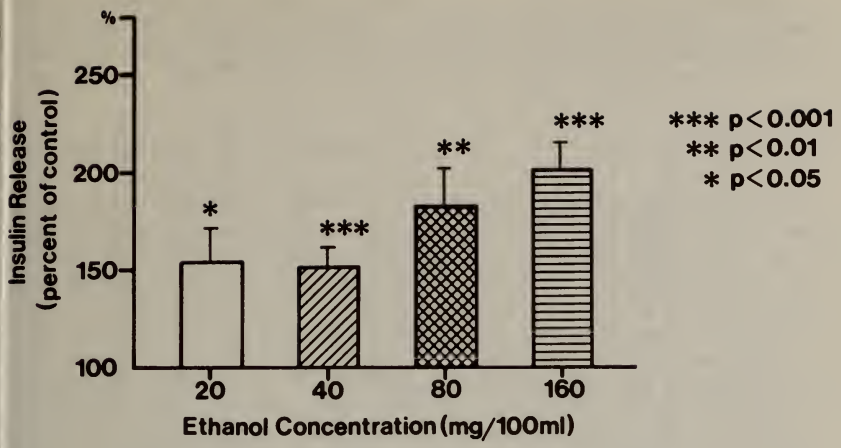

FIGURE 7.--Profile of insulin secretion from isolated pancreatic islets perfused with varying concentrations of ethanol

are not involved. However, propranolol decreased the influence of ethanol on insulin secretion. These studies show that ethanol directly induces insulin secretion, probably by acting on the β receptors of the β cells of the islets of Langerhans.

In constrast to our studies are those of Samols and Stagner (1980), who used the isolated perfused pancreas of the dog, and Patel and Singh (1979) who used rat isolated-islet incubation, in which they reported decreases in insulin secretion in response to very high concentrations of ethanol. In addition, the isolated islet incubation preparation of Patel and Singh (1979) could have missed the peak responses to ethanol due to their relatively long-term incubations, or an inhibitory factor could have been released into the media.

GLUCAGON AND SOMATOSTATIN

Glucagon, released from the α cells of the islets of Langerhans, increases when ethanol is administered IV (Palmer and Ensinck 1975) during the fasting state and is elevated in the fasted alcoholic human (Joffe et al. 1977). One exception occurred in the study of Tiengo et al. (1981), who used the isolated perfused rat preparation; there was no change in the glucagon levels, although the plasma insulin levels were decreased. The increase in glucogon levels in response to ethanol in the fasted state may be due to the removal of the insulin-inhibitory influence on glucagon since the insulin was generally depressed in these studies, or it could have been due to a direct effect of ethanol on the α cells of the islets of Langerhans. There are only a few studies on the effects of ethanol on glucagon secretion in the

TABLE 3.--Insulin secretion from perfused pancreatic islets

Agents in the perfusates	Increments of insulin secretion as percent of control*
Ethanol 80 mg percent	176±13
Epinephrine 9.2 μg/ml	123±21†
Propranolol HCl 30 μg/ml	100±11†
Phentolamine HCl 10 μg/ml	113±15†
Ethanol 80 mg percent + epinephrine 9.2 μg/ml	131±13‡ $p < .05$
Ethanol 80 mg percent + propranolol HCl 30 μg/ml	122±13‡ $p < .025$
Ethanol 80 mg percent + phentolamine HCl 10 μg/ml	179±18§

*Values represent mean ±SE values of four to six experiments.

†Not statistically significant when compared with the respective control as 100 percent.

‡Statistically significant at the respective levels by Student's *t*-test when compared with perfusion of ethanol alone.

§Not statistically significant by Student's *t*-test when compared with perfusion of ethanol alone.

fed or glucose-administered state, and they show a decrease or no change in glucagon levels. A thorough search of the literature has failed to uncover any studies in which the influence of ethanol on somatostatin levels has been determined.

FUTURE AREAS FOR RESEARCH

There is a desperate need for an animal model in which acute and chronic alcohol-induced alterations in gastrointestinal hormone release and their respective influences on each other can be determined precisely with consistent range of doses, duration of dose, and consideration of route of administration. Human subjects with prior exposure to ethanol or animals, such as dogs with Heidenhain pouches or gastric fistulas, which have been repeatedly exposed to damaging agents, cannot be considered naive subjects. More specifically, caution must be exercised in inferring that certain changes may have occurred in one study based on results from another study without actually measuring the changes produced by ethanol. For example, if serum gastrin levels are increased, one cannot conclude that gastric acid secretion has increased without actually measuring acid secretion since a primary defect producing hyposecretion, as frequently seen in chronic alcoholics, would result in removal of the usual acid inhibitory influence on gastrin. To assume that the possible release of certain hormones from the gastrointestinal tract by ethanol will release hormones from the pancreas can lead to considerable errors in judgment unless the hormone levels are actually measured.

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Alcohol Effects on Female Reproductive Hormones

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INTRODUCTION

An association between chronic alcoholism and menstrual irregularities was noted more than 160 years ago (Trotter 1813). Amenorrhea, infertility, and miscarriage, as well as gynecological problems that resulted in hysterectomy, are overrepresented in women with problems of alcohol abuse (Bourne and Light 1979; Hugues et al. 1980; Mello 1980). A specific association between moderate alcohol use and spontaneous abortions during the first and second trimesters of pregnancy has been reported (Harlap and Shiono 1980). Spontaneous abortions appeared to be specific to alcohol in the population studied and could not be explained by age, parity, race, marital status, smoking, or the number of previous spontaneous or induced abortions (Harlap and Shiono 1980).

A recent report indicates an impairment of hypothalamic-pituitary-ovarian function in 31 women with chronic alcoholism (Hugues et al. 1980). In the 13 alcoholic women of reproductive age, the hormonal disorders were due to a central defect, hypothalamic amenorrhea, anovulation, or inadequate luteal phase, reflecting a defect in tonic and cyclic control of gonadotropin secretion. Hugues et al. (1980) concluded that chronic alcoholism affects menstrual function at the hypothalamic-pituitary level. However, alcohol alone, liver damage, or nutritional status (or all three in combination) may contribute to the pathophysiology of the endocrine changes seen in female chronic alcoholics (Hugues et al. 1980). The lowest gonadotropin and estradiol levels were found in women with the most severe liver damage. Nutritional status has been shown to be important in the maintenance of normal menstrual cycles (Frisch and MacArthur 1974). Hugues et al. (1980) emphasize that the complications of chronic alcoholism in the women he studied make it difficult to ascribe these pathological findings to alcohol alone. However, these data argue for the importance of evaluating the effects of alcohol on

neuroendocrine function in nonalcoholic females of reproductive age whose liver function and nutritional status are normal.

There has been a surprising lack of studies of alcohol effects on the neuroendocrine system in women or in animal models. Cicero (1980b) comments, "There is little direct evidence regarding ethanol's effects on female reproductive function. Whether ethanol exerts any effects on the hypothalamo-pituitary axis, or at the level of the ovaries, remains a fertile area of investigation" (p. 553). Alcohol does appear to act as a gonadal toxin in female rodents (Cranston 1958; Eskay et al. 1981; Van Thiel et al. 1977, 1978). However, the significance of these data for women is unclear since there are major differences between human and rodent menstrual-cycle function (Yen 1980) as well as between rhesus monkeys and rodents (Knobil 1974, 1980). The physiology of the rhesus monkey is the most similar to human females, and it has long been the preferred model for studies of reproductive function (Knobil 1974, 1980; Pohl and Knobil 1982).

In an effort to clarify the effect of alcohol on female reproductive function, we have studied the acute effects of alcohol on neuroendocrine hormones in both postmenopausal and young women and in macaque monkeys. We also are examining the effects of chronic daily alcohol intoxication in a female monkey model of alcoholism. Some recent findings are summarized below.

ACUTE EFFECTS OF ALCOHOL ON YOUNG WOMEN

The acute effects of alcohol (and an isocaloric beverage) on plasma levels of prolactin, luteinizing hormone (LH), and estradiol, were examined in six normal, healthy women, aged 22 to 28 years (Mendelson et al. 1981). Women were selected on the basis of a complete physical and mental status examination and appropriate laboratory studies to rule out past or current history of physical or mental illness, alcoholism, or drug abuse. Subjects were fully informed of the nature of the study, were provided informed consent, and were paid for their participation. No subject had ever been pregnant, had a hysterectomy, or was using contraceptive medication or intrauterine device. All reported a past history of normal menstrual function, with an average cycle of 29 days, and a 4-day average duration of menstruation. Each woman participated in the study on the same day after onset of menstruation for two consecutive menstrual cycles (late follicular phase--8th to 10th day after the onset of menstruation) under alcohol- and placebo-control conditions.

Plasma prolactin, LH, and estradiol values before and after acute administration of alcohol or isocaloric beverage, are shown in figure 1 (Mendelson et al. 1981). All values are within normal range of levels for healthy, nonpregnant, and nonlactating human females. The menstrual cycle-phase was well matched across the two consecutive menstrual cycles since plasma hormone levels were virtually identical before administration of alcohol or isocaloric beverage.

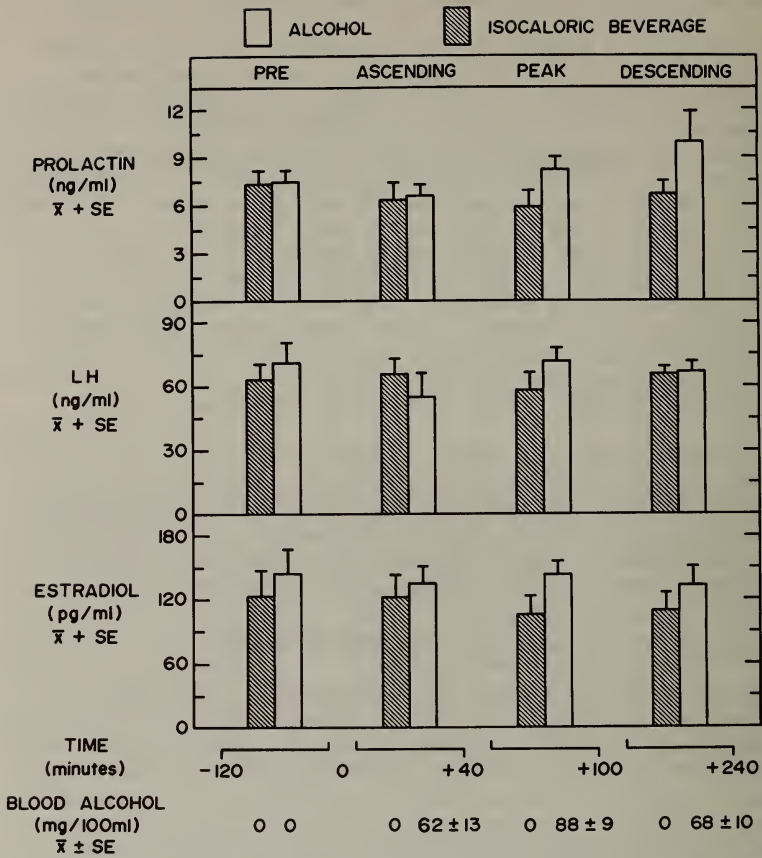

FIGURE 1.--Plasma prolactin, LH, and estradiol levels during the ascending, peak, and descending phases of the blood-alcohol curve and after administration of isocaloric beverage for six normal females. Computation of plasma prolactin, LH, and estradiol levels was carried out by obtaining the mean levels for all values determined on the ascending, peak, and descending phases of the blood-alcohol curve. From Mendelson et al. 1981. Copyright 1981 by the American Society for Pharmacology and Experimental Therapeutics.

There were no statistically significant differences in estradiol or LH following either alcohol or isocaloric beverage administration for times corresponding to the ascending, peak, or descending phase of the blood-alcohol curve. Moreover, there were no statistically significant differences in LH and estradiol following alcohol administration compared with values obtained after

isocaloric beverage. The only statistically significant finding was an elevation in prolactin levels, which occurred on the descending phase of the blood-alcohol curve. At this time, prolactin levels were significantly higher ($p < .05$) than at any other time after the administration of alcohol or isocaloric beverage. However, data from individual subjects revealed that prolactin elevations occurred only in three subjects, who also experienced nausea and vomiting during the descending phase of the blood-alcohol curve. Although prolactin levels were statistically significantly elevated at this time compared with prealcohol levels, they were still well within the normal limits for healthy nonlactating females.

ACUTE EFFECTS OF ALCOHOL ON ESTRADIOL IN MACAQUE MONKEYS

The acute effects of alcohol on 17-β estradiol, LH, and follicle stimulating hormone (FSH) were studied in sexually mature female macaque monkeys (six *M. mulatta*, one *M. nemestrina*; 4.0 to 8.5 kg) (Mello et al. 1982). Each hormone was studied at four phases of the menstrual cycle (menstruation, ovulation, midluteal phase, premenstruum). Progesterone was also studied at the midluteal phase. Alcohol doses of 1.5, 2.5, and 3.5 g/kg were compared with equal-volume isocaloric control solutions. Alcohol was prepared in a 25 percent solution (v/v) and administered through a pediatric nasogastric tube. Monkeys were fasted for 18 hours to ensure uniform absorption of alcohol from the small intestine. Peak blood-alcohol levels ranged between 150 and 325 mg/dl. Monkeys appeared severely intoxicated at alcohol doses of 2.5 and 3.5 g/kg.

Before and after acute alcohol (or sucrose) administration, integrated plasma samples were exfused continuously, and aliquots were collected every 30 minutes (Bree et al. 1982). Plasma levels of 17-β estradiol were determined in duplicate by radioimmunoassay (RIA) using a modification of the procedure of Hotchkiss et al. (1971). Intra and interassay coefficient of variables were 6 percent and 19 percent.

Acute alcohol intoxication did not affect plasma estradiol levels in female macaque monkeys (Mello et al. in press [b]). There was no correlation between plasma estradiol levels and the ascending and peak blood-alcohol levels. Moreover, estradiol levels did not decrease as a function of increasing alcohol doses at any phase of the menstrual cycle. Comparison of each postalcohol sample with the prealcohol sample for the monkeys showed no statistically significant differences. Comparison of each postalcohol sample with the postsucrose samples for the monkeys also showed no statistically significant differences. These data are consistent with our observations of human females (Mendelson et al. 1981).

We expected that acute doses of alcohol would produce a decrease in estradiol comparable with that seen in the male hormone, testosterone. In human males, acute doses of alcohol sufficient to produce peak blood-alcohol levels of 109 mg/dl resulted in a significant dose-dependent decrease in plasma testosterone (Mendelson et al. 1977). Single doses of alcohol have consistently resulted in dose-dependent decreases in plasma testosterone in rodents (see Cicero 1980a,b). It was surprising to find that even very high doses of alcohol, sufficient to produce blood-alcohol levels of 150 to 325 mg/dl, did not suppress estradiol in females since estradiol production requires essentially the same biosynthetic pathways as testosterone production in males.

To ensure that the absence of acute alcohol effects on estradiol in female monkeys did not reflect some unrecognized species difference, control studies of the acute effects of alcohol (2.5 and 3.5 g/kg) were conducted in male macaque monkeys. Preliminary findings from ongoing studies have shown a highly significant alcohol dose-related decrement in male macaque plasma testosterone levels. Prealcohol control values, averaging between 1,000 and 1,400 ng/dl fell to 200 to 300 ng/dl within 210 minutes after acute alcohol administration. These data indicate that alcohol effects on testosterone in macaque males are comparable with those consistently observed in other species (Cicero 1980a,b).

ACUTE EFFECTS OF ALCOHOL IN POSTMENOPAUSAL WOMEN

Pulsatile release of LH from the pituitary is modulated by adrenergically controlled LRF (luteininzing releasing factor) secretion from the hypothalamus (Yen et al. 1972; Naftolin et al. 1972). In premenopausal women, estrogen inhibits the dopaminergic induction of LRF release (Schneider and McCann 1970). Postmenopausal females do not have significant estrogen feedback control of LH secretion; therefore, alcohol effects on LH can be examined directly in the absence of estrogen modulation. Postmenopausal females are also more sensitive to the inhibitory action of drugs on adrenergic and dopaminergic pathways in the brain (Grossman et al. 1981). Consequently, if alcohol did inhibit LH, it would be more likely to be detected in postmenopausal females than in women of reproductive age.

We studied five healthy adult females (mean age 57) after complete cessation of normal menstrual cycles for at least 1 year. All women were in good health and reported normal menstrual function before the onset of menopause and no past history of gynecological disease, alcoholism, or drug abuse. Each woman was studied on two occasions, and either alcohol (2.2 ml/kg of body weight) or isocaloric beverage was administered orally after a 12-hour fast. Peak blood-alcohol concentrations averaged 94±8 mg/dl (SE) 90 minutes after alcohol intake. Blood specimens were collected from an indwelling intracatheter during consecutive 30-minute intervals for a total of 6 hours. Plasma LH concentrations were measured in duplicate from each 30-minute plasma sample

with a double-antibody RIA (Midgley 1966). Plasma LH (LER 907 ng/ml) values before (-120 minutes) and after (+300 minutes) acute alcohol intake ranged from 294.2±49.7 (SE) to 211.8±21.7 (SE).

No statistically significant changes in plasma LH occurred after alcohol administration in comparison with prealcohol LH values. There were no statistically significant differences between plasma LH after alcohol in comparison with LH levels after intake of the isocaloric control beverage. These data do not support the hypothesis that alcohol administration suppresses plasma LH in postmenopausal females. These findings are consistent with our observations in women of childbearing age (Mendelson et al. 1981).

EFFECTS OF CHRONIC ALCOHOL SELF-ADMINISTRATION IN THE FEMALE RHESUS MONKEY

Ongoing behavioral studies of the effects of chronic alcohol self-administration in female rhesus monkey have revealed a profound disruption of menstrual cycle regularity in animals who self-administered ethanol in doses of 3.5 g/kg/day and above. Monkeys were trained to self-administer food (1-g banana pellets), then were implanted with a venous catheter using aseptic procedures, and allowed to self-administer alcohol intravenously (IV). Alcohol (0.12 g/kg/injection) and food, both self-administered, were maintained under a second-order schedule of reinforcement (FR4 [VR 16:S]), which requires an average of 64 responses for each food pellet or alcohol injection. Persistent amenorrhea has been observed in monkeys that take stable high doses of alcohol. Alcohol was usually introduced shortly before the onset of menstruation, and no subsequent menses were observed.

One female rhesus monkey self-administered an average of 3.5 g/kg/day of alcohol for more than 3 months. Food intake varied as a function of intoxication, and the monkey lost 1.37 kg during 3 months of alcohol self-administration. This monkey died suddenly after 92 consecutive days of IV self-administered ethanol. The cause of death was not readily apparent from necropsy. The most parsimonious explanation is severe respiratory center depression as a consequence of ethanol overdose.

Several pathological changes in the reproductive system were observed upon necropsy. The uterus was markedly atrophied, consistent with changes usually observed after severe estrogen deficiency. Figure 2 is a low-magnification (×100) photomicrograph of the uterus, showing an atropic endometrium. There are few glands, and the endometrial stroma is dense. Figure 3 is a high magnification (×720) of a portion of the endometrium shown in figure 2. There is artifactual separation of the luminal epithelium for the underlying stroma. A segment of a single inactive gland is present in a dense population of small stromal

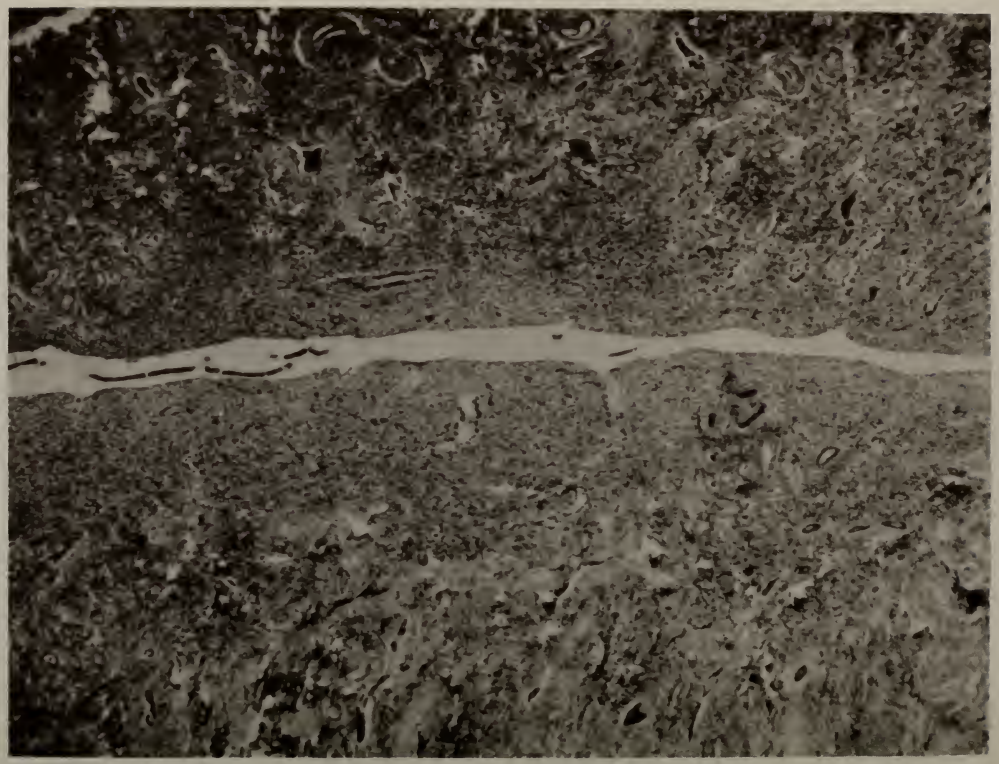

FIGURE 2.--Low-magnification photomicrograph of the uterus (×100). From Mello et al. in press (a). Reprinted with permission from Science. Copyright 1983 by the American Association for the Advancement of Science.

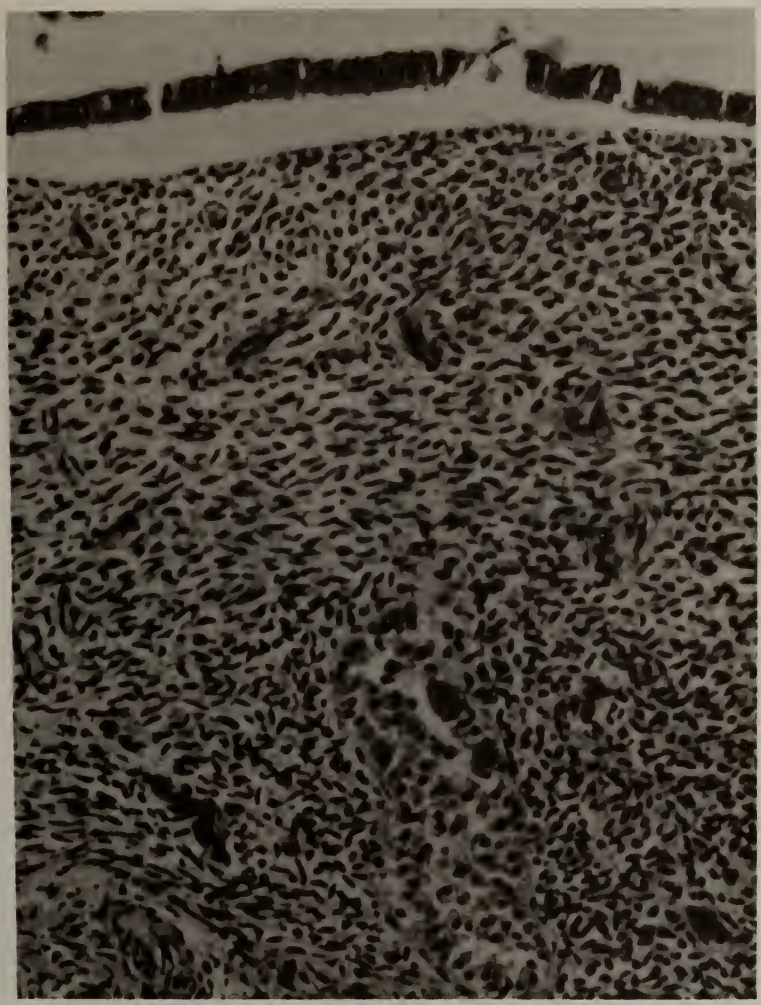

FIGURE 3.--High magnification of a portion of the endometrium (×720)

cells. Figure 4 shows the right ovary containing multiple Graafian follicles (×100). There were no corpora lutea present in either ovary. Figure 5 is a low-magnification (×100) photomicrograph of the adenohypophysis. The gland is quite cellular in appearance due to the presence of numerous small cells. Figure 6 is a high magnification (×720) of a portion of the adenohypophysis, which shows many small cells with small hyperchromatic nuclei scattered among the larger, polyhedral, parenchymal cells. The small cells resemble nucleated erythrocytes and thus may possibly represent extramedullary hematopoiesis, but their origin was not determined. However, no such cells were found in the liver or spleen, a much more common site for extramedullary hematopoiesis in severely anemic animals. The absence of similar cells in liver and spleen make it highly unlikely that the pituitary lesion represents extramedullary hematopoiesis. We postulate that the pituitary abnormalities are a very unusual degenerative lesion, possibly produced by prolonged, high-dose IV ethanol self-administration. It is also possible that a derangement in pituitary gonadotropin secretion induced the abnormalities observed in the ovary and uterus.

Analysis of estradiol and LH before and after initiation of chronic alcohol self-administration indicated that LH levels were within normal range (16 to 39 ng/ml) even after 92 days of high-dose alcohol intake. Only one low LH value (8 ng/ml) was observed on day 20 of the first menstrual cycle after alcohol administration began. Estradiol levels ranged between 39 and 144 pg/ml during the first 40 days of alcohol self-administration.

These findings are consistent with data obtained in studies of chronic ethanol administration to rodents (Van Thiel et al. 1978; Gavaler et al. 1980), where decreased gonadal mass was due to the absence of corpora lutea and estradiol levels were in the normal range for the early follicular phase.

LH levels in the female rhesus monkey before death were within normal ranges usually found before and after the preovulatory LH surge. Since daily LH levels were not determined, a complete or partial LH surge may have occurred but was not detected. However, the absence of corpora lutea strongly suggests that ovulation did not occur whether or not there was an LH surge. Moreover, it remains to be determined whether the cellular changes observed in the adenohypophysis have any relationship to possible alterations in LH secretory activity.

Further elucidation of ethanol effects on pituitary gonadal hormones and menstrual-cycle function in the female is clearly necessary. However, it is unlikely that the most relevant data for humans will be obtained in studies with infrahuman species other than monkey. The rat requires a neural signal from the preoptic-anterior hypothalamus for initiation of the preovulatory LH surge (Weick et al. 1973; Karsch et al. 1973a). This diurnal signal that is essential for ovulation in the rat (Sawyer 1969) is blocked by the administration of pentobarbital, but barbiturates do not inhibit the estrogen-induced LH surge in the rhesus monkey

FIGURE 4.--Low-magnification photomicrograph of the ovary (×100). From Mello et al. in press (a). Reprinted with permission from Science. Copyright 1983 by the American Association for the Advancement of Science.

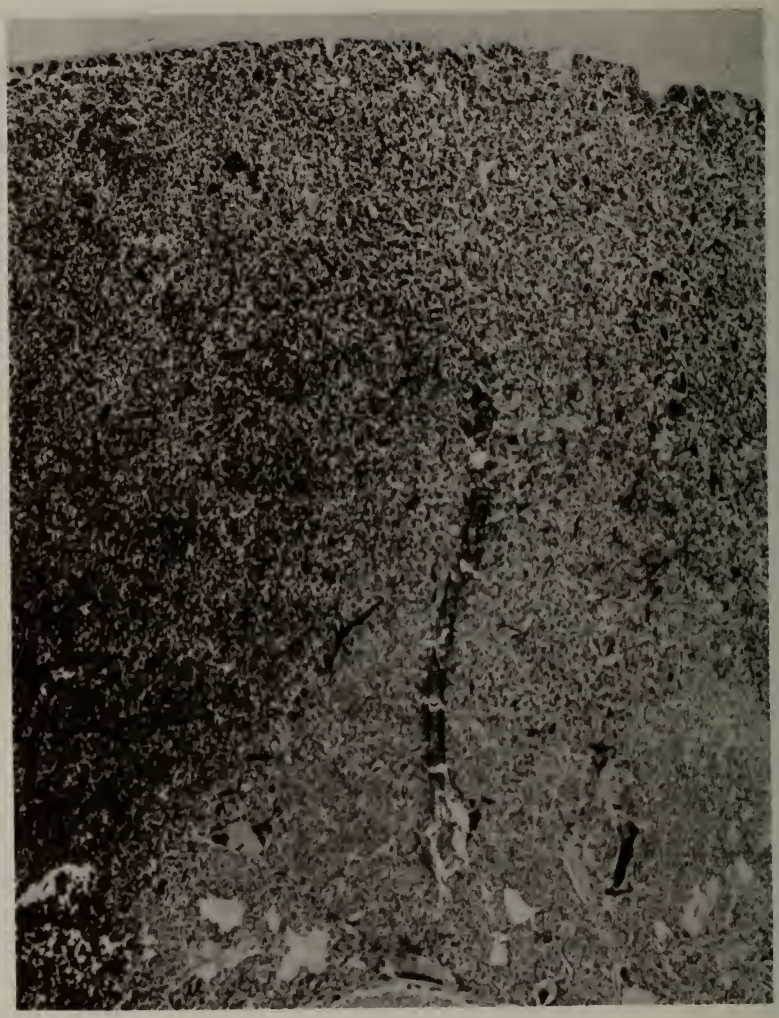

FIGURE 5.--Low-magnification photomicrograph of the adenohypophysis (×100)

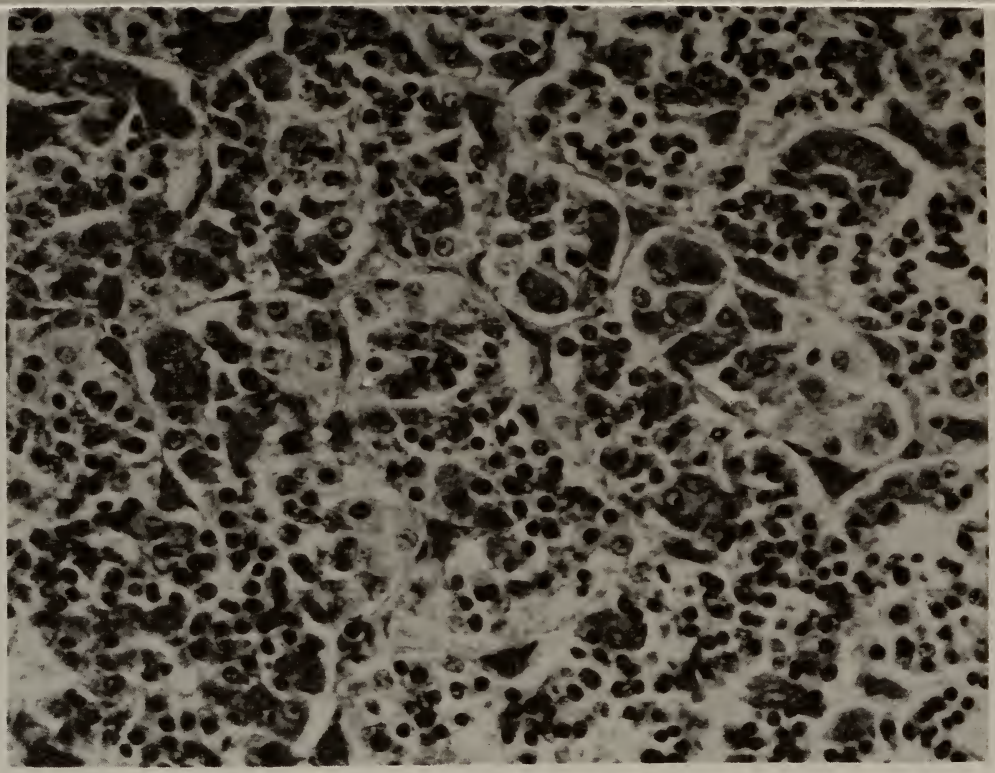

FIGURE 6.--High magnification of the adenohypophysis (×720).
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(Knobil 1974). Since barbiturates and ethanol have many common effects in the central nervous system (CNS), it is postulated that the effects of these drugs on the hypothalamic-pituitary axis in rat may differ from the effects in the rhesus monkey or humans. There are also data that indicate that application of ethanol effects on the male rodent hypothalamic-pituitary axis may be limited for understanding the effects of alcohol on pituitary-hypothalamic function in human males. These limitations may be especially important for understanding ethanol-induced alterations in steroid feedback regulatory effects on the hypothalamus in human males. The adult male rat does not show an LH surge after estradiol stimulation (Neill 1972), but adult male rhesus monkeys do (Karsch et al. 1973b). Finally, the findings of Knobil (1981), which support the hypothesis that "GNRH may play only a permissive, albeit obligatory, role in the control of gonadotropin secretion," in the rhesus monkey and humans, but not in rodents, strongly argue for more research on acute and chronic ethanol effects on the hypothalamic-pituitary-gonadal axis in monkeys and in human males and females.

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ROLE OF HORMONES IN METABOLIC TOLERANCE TO ETHANOL

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Overview

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In discussing the hormonal regulation of alcohol metabolism, it is important to recall the rate-limiting steps in ethanol metabolism. Regulation, hormonal or otherwise, is more effective when it occurs at these steps.

It is generally agreed that the alcohol dehydrogenase (ADH) pathway accounts for more than 70 percent of the metabolism of alcohol in the liver and that the first reaction, that of NAD + ethanol to yield acetaldehyde + NADH, catalyzed by ADH, constitutes the most important rate-limiting step in the elimination of alcohol. After attaining saturation at the ethanol site, both the amount of ADH and the rate of reoxidation of NADH (determining the concentration of NADH and NAD), are important rate-limiting steps (Plapp 1975; Higgins 1979; Israel et al. 1975; Crow et al. 1977). Which of these two predominates depends on the experimental conditions and the species studied. For example, in rodents under conditions of starvation the amount of ADH is markedly reduced, and thus it becomes an important rate-determining step (Lumeng et al. 1979). Similarly, in some rat strains, the synthesis (or degradation) of ADH is under strong control of testosterone, such that the metabolism of ethanol is substantially lower in males than in females (Rachamin et al. 1980). The rate of ethanol metabolism in males is greatly increased by castration and can be markedly reduced by further administation of testosterone (Rachamin et al. 1980; Mezey et al. 1980; Cicero et al. 1980). Females also respond to testosterone with a reduction in ADH activity and in the rate of ethanol metabolism (Cicero et al. 1980).

In humans the rate of ethanol elimination is not substantially different in females than in males (Wallgren and Barry 1970); females, however, have less body water and thus a smaller volume for ethanol distribution, so that ethanol is expected to disappear from the blood more quickly than in males. This should, however, not be taken as an indication of a faster rate of ethanol metabolism. Similarly, men who present the "atypical" or Oriental form of ADH, showing in vitro activities about fourfold that of the "typical" enzyme, do not metabolize alcohol at significantly higher rates (Edwards and Price-Evans 1967; von Wartburg and Schurch 1968). Although it can be argued that we do not know the actual levels of NAD^+ and NADH in the livers of humans metabolizing alcohol, in comparison with their $\underline{K}_m$ and $\underline{K}_i$ values

for ADH, respectively, this argument implicitly indicates that the rate of reoxidation of reducing equivalents is an important rate-determining factor. On the basis of data by Lester and Keokosky (1967) and of Videla et al. (1975), Higgins (1979) has calculated that the metabolism of ethanol to acetate, in a variety of animal species, requires 67 to 80 percent of the oxygen used by the liver. Oxygen consumption by the liver is, in turn, a measure of the capacity of that organ to oxidize reducing equivalents. Thus, on simple stoichiometric grounds, the rate of ethanol metabolism should follow the basal metabolic rate, i.e., the latter constitutes an important rate-determining step in the metabolism of ethanol.

The relationship between alcohol metabolism and basal metabolic rate in 10 different species, including man, has an $r = 0.95$ (Videla et al. 1975), thus indicating that 90 percent (r^2) of the variability in ethanol metabolism in different species can be accounted for by the capacity to utilize oxygen (and reducing equivalents). It would appear that the ratio ADH/QO_2 determines which of the two steps, reoxidation or ADH activity, predominates as the most important rate-limiting factor. In small animals such as rodents, in which ADH is not in large excess, the basal metabolic rate and liver QO_2 are high (Videla et al. 1975). In these ADH constitutes an important rate-limiting step. On the other hand, in larger animals such as the horse, basal metabolic rate is low (Videla et al. 1975), and ADH activity is very high (Plapp 1975). In these animals reoxidation of reducing equivalents is likely to be the major determinant of the rate of ethanol metabolism.

Some of the chapters in this book address the role of hormones at the different regulatory steps of ethanol metabolism. Since glucagon is not covered, its role is emphasized here. Along with thyroid hormones, glucagon is a typical calorigenic hormone. Acute glucagon infusion increases the rate of liver oxygen consumption and the rate of ethanol metabolism in the cat in vivo (Larsen and Krarup 1974). Since ethanol elevates glucagon levels in vivo (Jauhonen 1978; Tiengo et al. 1974; Potter and Morris 1980), it is likely that ethanol per se plays a role in stimulating the rate of its own metabolism. Such an effect may contribute to the increased rates of ethanol metabolism that some investigators have observed at high blood-ethanol concentrations (Salaspuro and Lieber 1978; Pikkarainen and Lieber 1980; Mezey in press). The author has done experiments in which administration of somatostatin (0.9 mg/kg) in divided doses over 4 hours was shown to significantly reduce the rate of ethanol metabolism in female Sprague-Dawley rats after a 2.5-g/kg dose (control 370±7 [Rachamin 1980]; somatostatin 319±7 [Rachamin 1980] mg/kg/hour; $p < .001$, mean ±SEM). Somatostatin is a powerful inhibitor of glucagon release from the pancreas.

The above discussion suggests that hormonal effects at either level of ADH reoxidation may contribute to the rate of ethanol elimination. Mezey elegantly presents the complex interactions

on ethanol metabolism of stress and of hormones derived from the different hypothalamic-hypophyseal-peripheral axes of the sympathetic nervous system. An example of interaction at the two rate-determining levels is provided by thyroid hormones, which increase the rate of liver oxygen consumption but also inhibit ADH and are required for the operation of one of the mitochondrial NADH-transporting shuttles. Mezey also provides interesting information on the inhibitory effects on ethanol metabolism by testosterone and its metabolite, dihydrotestosterone, acting apparently by different mechanisms. Von Wartburg introduces exciting findings in the immunochemical detection of ADH in some neuronal systems, particularly in Purkinje cells in the cerebellum, and in the islets of Langerhans in the pancreas. Recent studies on the role of atypical ADH and variability in low K_m aldehyde dehydrogenase are also discussed. Thurman et al. present studies showing the permissive role of thyroid hormones in the swift increase in alcohol metabolism (SIAM) induced by a single large dose of ethanol. These studies, along with others in the literature, suggest that SIAM and chronic metabolic tolerance might occur through similar mechanisms. Sturtevant presents interesting data showing marked circadian variations in the rate of ethanol disappearance from blood and the rate of metabolism. In normal animals liver ADH activity also shows marked circadian variation, but the microsomal ethanol-oxidizing system (MEOS) showed virtually no circadian rhythm. The rhythm in MEOS, however, appeared in animals that were fed alcohol (15 percent) for 22 weeks and was out of phase with regard to the ADH rhythm.

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Variability of Human Alcohol Metabolism

Jean-Pierre von Wartburg

INTRODUCTION

It is well established that only a few percent of a population drink one-third to one-half of the total alcohol consumed in that population. It is also known that most of the people in an alcohol-consuming society drink little or moderately during their lifetime without developing any major alcohol-related problems. Finally, genetic studies show that not everyone is at equal risk for developing alcohol-related disorders that might be termed alcoholism (Schuckit 1980). Thus, the question arises as to whether genetically predetermined differences in alcohol metabolism among individuals might influence the transition from moderate low-risk to excessive high-risk drinking and whether such differences are predisposing factors for alcohol-related organic injury. In this chapter I advance the hypothesis that indeed quantitative and qualitative differences in alcohol metabolism may offer partial answers to these questions. The main emphasis is on the large variation in alcohol and acetaldehyde metabolism in humans and on the possible mechanisms that underlie the largely variable acetaldehyde levels in blood and probably also in the endocrine organs.

VARIABILITY OF ALCOHOL METABOLISM

For a long time it has been the classical view that blood-alcohol removal rates are independent of the blood-alcohol concentration and are similar in most individuals. Recent observations show that alcohol metabolism is quite variable among individuals, reflecting the astonishing variations of the enzyme systems that are responsible for ethanol and acetaldehyde oxidation (von Wartburg 1980a,b,c, 1982).

Several research groups have observed that the activity of alcohol dehydrogenase (ADH) in the human liver shows a considerable interindividual variation (von Wartburg et al. 1965; Moser et al. 1968; Schenker et al. 1971; Li and Magnes 1975). It is generally accepted that in humans it is mainly the activity of liver ADH that determines the rate of ethanol metabolism, although it is

clear for theoretical reasons that the rate of coenzyme (NADH) reoxidation, is also co-rate-limiting (Plapp 1975; Higgins 1979). Hence, the variation in ethanol metabolism mainly reflects the wide range of the catalytic properties of human ADHs. This, in turn, is easily explained by the many multiple molecular forms observed for this enzyme, although their nature is by no means fully understood (von Wartburg et al. in press).

The many forms are due to the existence of isoenzymes. They are generated by the random combination of three different subunits (α, β, γ) to the six possible dimeric forms. Additional enzyme forms are due to enzyme polymorphisms. In the gene loci coding for the β and γ polypeptide chains, allelic genes occur yielding γ_1 and γ_2 subunits (von Wartburg 1982; Smith et al. 1973a,b; Li 1977). Several polymorphisms seem to occur at the gene locus coding for the β subunits; in addition to the normal β_1 subunit, an "atypical" β_2 subunit (β_{Berne}) (von Wartburg et al. 1965; von Wartburg and Schurch 1968) or a $\beta_{Indianapolis}$ subunit (first discovered in Indianapolis) can be formed (Bosron et al. 1980; Li 1982). In addition to these forms, further ADH forms have been identified. π-ADH is found in all individuals but to a variable extent (Li and Magnes 1971). Finally, further forms that show cathodic mobilities on electrophoresis (X-enzyme) have been described (Pares and Vallee 1981). No knowledge of their structure or genetic control is available.

Thus, a number of factors determine the remarkable variability of ADH in human organs: (1) the occurrence of genetically determined isoenzymes and enzyme polymorphisms; (2) variation in the gene expression due to developmental stages; (3) organ specificity of the isoenzyme pattern of the various organs, such as liver, gastrointestinal tract, kidney, lung, pancreas, brain, endocrine organs; and (4) pathological alterations in the tissue.

Besides this variability of the main enzyme of alcohol metabolism--i.e., ADH--a microsomal ethanol-oxidizing system (MEOS) may be induced by chronic exposure to alcohol or some drugs (Lieber 1977) and thus add to the variability in blood-alcohol removal (Korsten et al. 1975). Both the Michaelis-Menten constants as well as the specific activities of these alcohol-metabolizing enzymes vary tremendously (von Wartburg 1982; Li 1977; Buhler and von Wartburg 1982) so that the contribution of any given enzyme form to the overall metabolism in the liver and other organs will depend on the momentary alcohol concentration at that site.

According to the classical work of Widmark and others blood-alcohol elimination is approximately linear and remains independent of ethanol concentration until very low concentrations are reached. However, this concept of linearity has been challenged by a number of researchers who observed concentration dependence of blood-alcohol clearance. Consequently, pharmacokinetic models for ethanol elimination that follow Michaelis-Menten kinetics have been proposed (Wilkinson 1980; Wilkinson et al. 1980). However, these models assume only one Michaelis-Menten constant.

Further, they do not take into account the possibility that relatively high alcohol concentrations are reached in the portal vein during absorption from the gastrointestinal tract, which could lead to saturation of high K_m isoenzymes. Considerable amounts of alcohol could be removed by such a first-pass elimination in the liver, which would not appear in the peripheral blood and would make the area under the blood-alcohol curve disproportionately small (von Wartburg 1982). Since the exposure of many critical sites, such as the brain and endocrine glands, to the direct effects of ethanol depends on the kinetics of blood-ethanol removal, improved pharmacokinetic models should be developed on the basis of the knowledge of the catalytic characteristics of the enzymes involved.

ACETALDEHYDE

So far we have mainly discussed quantitative aspects of the variability of alcohol metabolism. We can also envision differences of qualitative nature--namely, a variability in the levels of the first intermediary metabolic product of ethanol oxidation, i.e., acetaldehyde. In view of acetaldehyde's many possible toxic effects, it is important to know where and at what concentrations this compound occurs during ethanol metabolism. It is reasonable to assume that steady-state levels of acetaldehyde at any given place in the body are determined by the balance between its rate of production and removal.

Acetaldehyde disposal takes place mainly by metabolism to acetate, whereby aldehyde dehydrogenase plays the major role (von Wartburg 1980a, 1982; Lindros 1978). In comparison with human ADH, surprisingly little is known about human aldehyde dehydrogenase and its genetic control. Several multiple molecular forms have been described and characterized. Thus it is likely that acetaldehyde dehydrogenase activities show the same large variability as that found for ethanol oxidation (von Wartburg 1982; Hopkinson 1982; Harada et al. 1982, in press; Agarwal et al. 1982; Inoue and Lindros 1982). In addition, an enzyme polymorphism has been found in Oriental populations: about 50 percent of the individuals lack one enzyme form, which seems to be the low K_m mitochondrial enzyme (Goedde et al. 1979, in press). A very slow acetaldehyde metabolism would be expected in such individuals, and indeed extremely high acetaldehyde blood and breath levels have been observed as a consequence of this enzyme polymorphism (von Wartburg et al. in press).

For further discussion, I define three different levels of possible blood acetaldehyde in humans: (1) the normal levels; (2) the levels for acute aldehyde syndrome, which are extremely high; and (3) the levels for chronic aldehydism, which are only slightly increased values.

NORMAL ACETALDEHYDE LEVELS

For many years methodological difficulties have prevented accurate determinations of acetaldehyde concentrations in blood or tissue samples. Now several methods are available that give comparable results, and agreement has been reached on the actual levels among various groups of researchers (von Wartburg et al. in press; Eriksson in press). The range of normal acetaldehyde concentrations depends on the alcohol dose. With ethanol ingestions of 0.25 to 0.75 g/kg body weight, acetaldehyde levels beyond the limit of detection (ca 0.5μM) up to 2 to 3μM were found.

ACUTE ALDEHYDISM LEVELS

In individuals with acute aldehyde syndrome, acetaldehyde concentrations are 10 times normal or more. This phenomenon is observed in Orientals who lack the low K_m mitochondrial aldehyde dehydrogenase (Mizoi et al. in press; Harada et al. 1982, in press) or after treatment with aldehyde dehydrogenase inhibitors, such as disulfiram (Maring et al. in press). Symptoms such as heavy facial flushing, tachycardia, hypotension, headache, nausea, vomiting, muscle weakness, and sleepiness may occur, and the degree of the symptomatology seems to depend on acetaldehyde levels. These symptoms are clearly aversive and would be expected to prevent such individuals from drinking. Interestingly, only a small percentage of those who lack the mitochondrial isoenzyme has been found among Japanese alcoholics as compared with approximately 50 percent in the whole population (Harada et al. 1982, in press; Yoshihara et al. in press). This finding is well in keeping with the hypothesis that a genetic hypersensitivity toward the effects of alcohol prevents these individuals from high-risk drinking.

CHRONIC ALDEHYDISM LEVELS

In individuals with chronic aldehydism, acetaldehyde concentrations are only slightly elevated, about two to five times the normal concentrations (Korsten et al. 1975; Lindros et al. 1982; Maring et al. in press). Such levels might result from specific isoenzyme patterns of alcohol and aldehyde dehydrogenase and/or the induction of MEOS, leading to slightly increased acetaldehyde production combined with a slightly slower removal rate. Higher acetaldehyde levels in the tissue could predispose one to greater intracellular cytotoxic effects, thus leading to increased organic damage. In this context it is of interest that high frequencies of atypical ADH have been observed in alcohol liver disease (Kamaryt and Martinek 1979; Salaspuro et al. 1982).

ADH IN HUMAN TISSUES

Many organs and the endocrine glands are known to be affected by chronic alcohol intoxication. In some instances it has been observed that acetaldehyde is at least as effective as ethanol in inducing intoxication. As discussed above, variable concentrations of acetaldehyde in blood could lead to different amounts of this toxic compound's diffusing into tissues. On the other hand, the presence of ADH and/or MEOS in such organs and glands could lead to the intracellular formation of acetaldehyde. Hence, we undertook an immunohistochemical study of the localization of ADH in human tissues (Buhler et al. 1982a, in press). Antibodies against a mixture of the pyrazole-sensitive human liver ADH were elicited in rabbits. Protein A-peroxidase with diaminobenzidine as substrate was used to detect anti-ADH bound to the enzyme in thin sections of tissues. With this method at least some ADH could be detected in all tissues investigated. The control sera were always negative. In some organs certain cell types stained more strongly than others within the same organ, indicating that the enzyme is unevenly distributed in many organs. Such uneven distribution was found in the kidney, gastrointestinal tract, and liver. This is an important observation because it means that ADH may be present in high concentrations in only a few cells of an organ although the overall activity of ADH in that tissue may indicate that only traces of ADH are present. High concentrations of acetaldehyde at these specific locations might then lead to discrete cell injury.

In the brain, for instance, only some neurons showed strong staining in sections of the cerebral cortex, cerebellum, and hypothalamus. Particularly, some Purkinje cells in the cerebellum stained very strongly. It is not yet possible to specify the neurons with high ADH content, but some neurons also stained in the infundibular stalk of the pituitary gland.

The testes are affected by alcohol, and the involvement of ADH has been suggested (Cicero et al. 1980; Van Thiel et al. 1980; Ylikahri et al. 1980). We found most of the ADH in the Leydig cells, followed by the seminiferous epithelium, with the least ADH in the interstitial cells. These results agree well with the proposed role of acetaldehyde formation in that organ. Further ADH was found in the prostate and in the epididymis.

In the ovary and uterus only weak staining was observed. This observation is again in agreement with the conclusion that ADH plays no role in the toxic effects of ethanol on these organs (Poso and Poso 1981).

In the pancreas both the exocrine and endocrine functions are affected by alcohol. Interestingly, in vitro experiments with rats show that acetaldehyde and acetate have marked effects on insulin and glucagon release (Calderon-Attas et al. 1980; Tiengo et al. 1981), and traces of ADH have been found in homogenates of whole pancreatic tissue (Clemente et al. 1977; Schmidt and

Schmidt 1960). Although ADH was found in both the exocrine and endocrine part of the pancreas, much more enzyme was observed in the islets of Langerhans, suggesting that ADH may be involved in the toxic effects of alcohol on pancreas.

In view of the toxic effects of acetaldehyde on adrenals in the rat, the possible formation of acetaldehyde from ethanol by ADH would be of special interest. However, the overall staining of the glands was weak, the adrenal cortex showing slightly more ADH than the medulla. Hence, it is more likely that the effects of acetaldehyde on the adrenals, such as the release of catecholamines, are mediated by blood acetaldehyde's diffusing into the glandular tissue.

In general we may conclude that specialized cells within endocrine organs may contain large amounts of ADH, even though the overall activity of the enzyme in the gland may be quite low. Hence, many of the organs could be direct targets of acetaldehyde during ethanol intoxication even at low blood-acetaldehyde levels.

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Circadian Rhythms in Ethanol Metabolic Rate

Ruthann P. Sturtevant

INTRODUCTION

For a diverse array of drugs, including ethanol, pharmacological response may vary considerably with the circadian metabolic phase of the recipient. Such findings have made it necessary to expand the focus of traditional biochemical, physiological, and pharmacological methods to include chronobiological considerations. Over the past decade, we have noted a linguistic explosion of such terms as chronotoxicology, chronopharmacokinetics, and chronotherapy, which seek to apply laboratory findings to the clinical setting.

The first report specifically directed toward the biorhythmicity of ethanol metabolism in human subjects was that of Wilson et al. in 1956. These workers reported that the blood-ethanol levels in nonfasted subjects who received repeated oral doses that were slightly greater than the ethanol metabolized, tended to rise during one-half of the 24-hour day and to fall during the other half. More recently, a number of workers have investigated the chronopharmacokinetics of human ethanol metabolic rate following single (Sturtevant et al. 1976; Minors and Waterhouse 1978) and repeated dosing (Sturtevant et al. 1978). The difficulties inherent in controlling standardized experimental conditions within a group of inebriated human subjects necessitated the selection of an appropriate small laboratory animal for characterizing the circadian variation of the ethanol metabolic rate.

Unlike the mouse, in which a time-invariant rate of ethanol clearance has been reported (Deimling and Schnell 1980), the rat proved to be a useful model species. Like humans, the rat displays a chronosusceptibility to ethanol. The rat's blood volume allows it to take repetitive 20-μl blood samples without undue stress. This permits individual metabolic rates to be assessed and obviates the need to pool samples for blood-ethanol determinations. In examining the basic experimental variables fundamental to our anticipated investigations of the chronopharmacokinetics

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of ethanol, we limited our studies to male rats to avoid possible variables associated with the estrus cycle.

Young male rats (stock COBS CD(SD)BR), were housed in climate-controlled environmental chambers. Our standard regimen for all initial studies included a 12-hour:12-hour light:dark (LD) cycle within the chambers and continuous availability of rat chow and tap water. When different groups of rats, each housed within a single chamber, were injected intraperitoneally (IP) with 1.5 g/kg ethanol (11.7 percent, w/v) at various times over the 24-hour span, peak blood-ethanol clearance rates, as determined by gas chromatography, consistently fell near the time of the dark-to-light transition (Sturtevant et al. 1981a). As seen in figure 1, a reversal of the clock hours of light and darkness effected a 180° phase reversal of the rhythm. The relationships between the timing of the rhythm and the phasing of the LD cycle were maintained, with the alternate periods of illumination and darkness providing the external cues for the phase timing. Persistence of the rhythm in the absence of such cues, i.e., during prolonged periods of constant light or constant darkness (Sturtevant 1980), provided evidence that the rhythm was of endogenous origin, encoded within the organism's genetic mechanism. It was cued or synchronized but not caused by external signals.

EXTERNAL EVENTS

As illustrated above, the LD cycle is generally found to be the dominant synchronizer of the sleep-wakefulness cycle in laboratory rodents. It overrides other external cyclic events, such as atmospheric pressure, temperature, or background noise levels, in maintaining the normal phase relationships among the multitude of metabolic processes that occur over the circadian time span. A metabolic disruption with desynchronization of internal events generally follows a sudden and drastic alteration in the sleep-wakefulness pattern. In the laboratory, we can bring this about simply by advancing or delaying the time of the light-to-dark change. In humans, an abrupt schedule change for the shift worker or the "jet lag" of the west-to-east airline traveler has similar effects. This desynchronization gradually lessens during the ensuing transition period as the normal metabolic relationships are reestablished.

When food availability is restricted to 4 hours/day, however, the feeding regimen overrides the LD cycle as the dominant rhythm synchronizer (Sturtevant and Garber 1980, 1981). Two groups of rats were maintained on opposite 12 hour:12 hour LD cycles such that the clock hours of light for one group corresponded to the hours of darkness for the other group. Food was presented to both groups simultaneously, and the uneaten pellets were removed 4 hours later. Thus, one group was eating at the normal (mid-dark) phase and the other during the mid-light phase, the most unnatural period for rats to feed. After

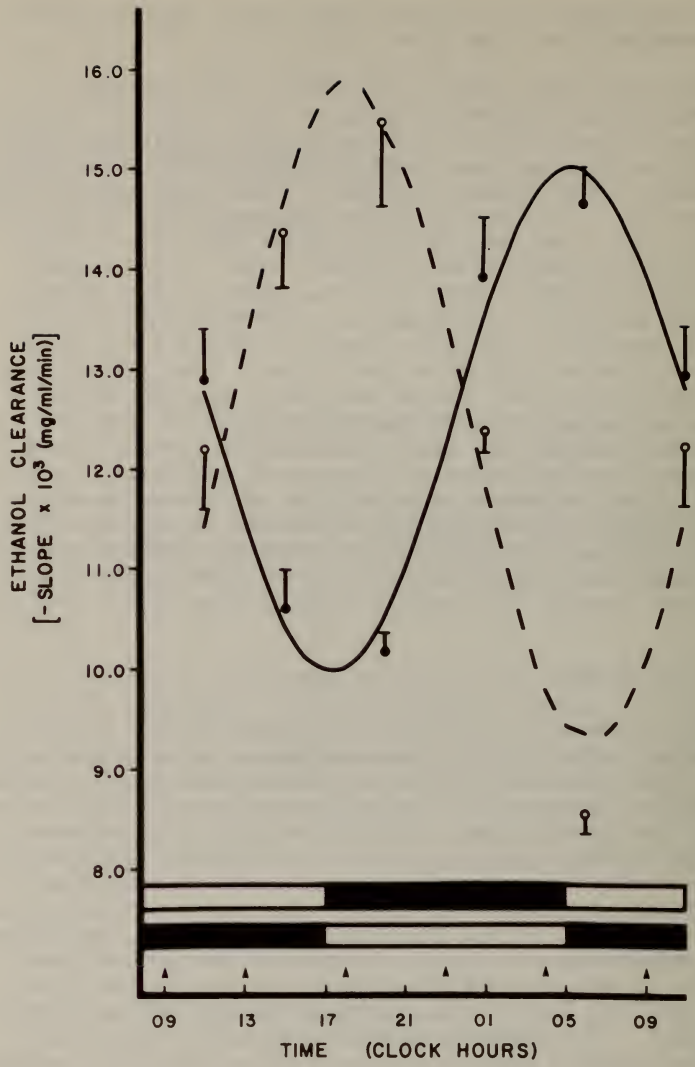

FIGURE 1.--Circadian rhythm in blood-ethanol clearance rates for two groups of ad-lib fed male rats maintained on opposite 12 hour:12 hour LD cycles (open and solid horizontal bars, respectively). Arrowheads indicate times of IP ethanol injections to subgroups (N = 8) of both groups. Clearance slopes ±SE, shown at sampling midpoint, are superimposed by the best fitting cosine curve. Group 1, solid line; group 2, dashed line. The upper and lower horizontal bars indicate the hours of light (open bar) and darkness (solid bar) for groups 1 and 2, respectively. From Sturtevant and Garber 1981. Copyright 1981 by J Nutr, American Institute of Nutrition.

a 4-week acclimatization to this regimen, the blood-ethanol clearance rate rhythms, which had been 180° out of phase during the ad lib feeding regimen (figure 1), were now virtually in phase, having been synchronized by the schedule of food availability (see figure 2).

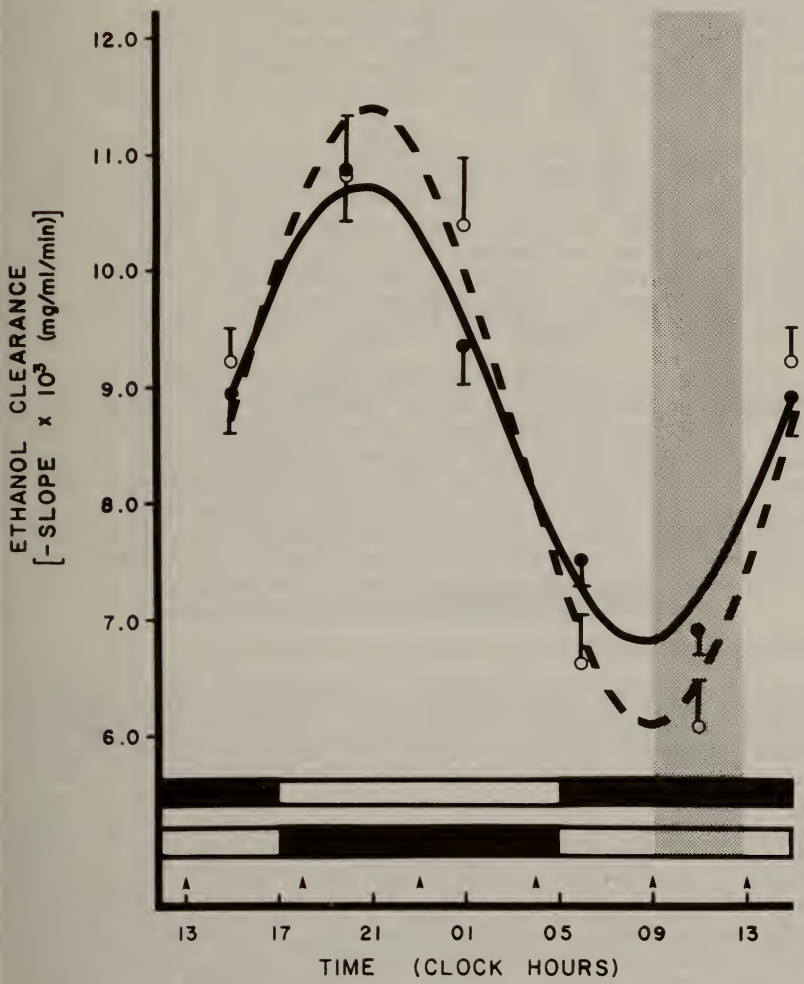

FIGURE 2.--Circadian rhythm in blood-ethanol clearance rates for two groups of food-restricted male rats. Hours of food availability indicated by vertical stippled bar. Details of figure as in figure 1. From Sturtevant and Garber 1981. Copyright 1981 by J Nutr, American Institute of Nutrition.

INTERNAL EVENTS

CORTICOSTEROID LEVELS

The circadian rhythm in blood-corticosteroid levels has been well documented for man and for various laboratory animals (for a review, see Sturtevant and Sturtevant 1981). Although this rhythm is remarkedly stable, a characteristic that has made it useful as a reference or internal marker rhythm to which the phasing of other physiological variables can be related, is that its phasing and amplitude can be affected by certain drugs. Ethanol, for example, has been shown to induce an acute elevation of corticosteroid levels in a number of species.

We investigated the effects of repeated IP doses of ethanol on the circadian excursions in plasma corticosteroids in three groups of 24-hour starved rats. The amplitude of the steroid rhythm (one-half of the difference between the highest and lowest 24-hour values) was unchanged from that previously reported for ethanol-naive rats. The acrophase (peak 24-hour value), however, was somewhat phase advanced and occurred during the mid-to-late light phase of the LD cycle (Sturtevant et al. 1981a).

In another study, we examined the effects of repeated doses of ethanol on blood-cortisol levels in five adult nonalcoholic human males. The phase relationships of the circadian cortisol and blood-ethanol clearance rate rhythms showed individual variation. The rhythms tended to be in phase in some subjects and out of phase in others (Sturtevant et al. 1981b). These studies clearly indicated that a single time point determination to evaluate drug-induced effects on blood-steroid levels may court serious error in interpretation.

FEMALE HORMONES

To date, we know of no reports concerning chronovariability of ethanol metabolic rates in female rats. To assess the effects of female hormone-associated cyclicity on those rates, 48 1-month-old, virgin Charles River rats were divided into six groups of eight each. All groups were maintained on a 12 hour:12 hour LD cycle, with rat chow and water freely available. When the rats were 8 week old, one group was injected IP with 1.5 g/kg ethanol 1 hour after the onset of the light phase. Beginning 60 minutes later, eight 20-μl samples of tail vein blood were taken from each animal at 20-minute intervals. The remaining groups were injected and sampled successively at 4-hour intervals over a single 24-hour span. Figure 3 shows the slopes of the blood-ethanol clearance curves superimposed by the best fitting cosine curves determined by the methods of Nelson et al. (1979) and Cornelissen et al. (1980). In addition, the 24-hour variation in rate of ethanol metabolism was determined by extrapolating the clearance slope to the abscissa to yield the theoretical time of zero blood concentration. The dose was divided by this time

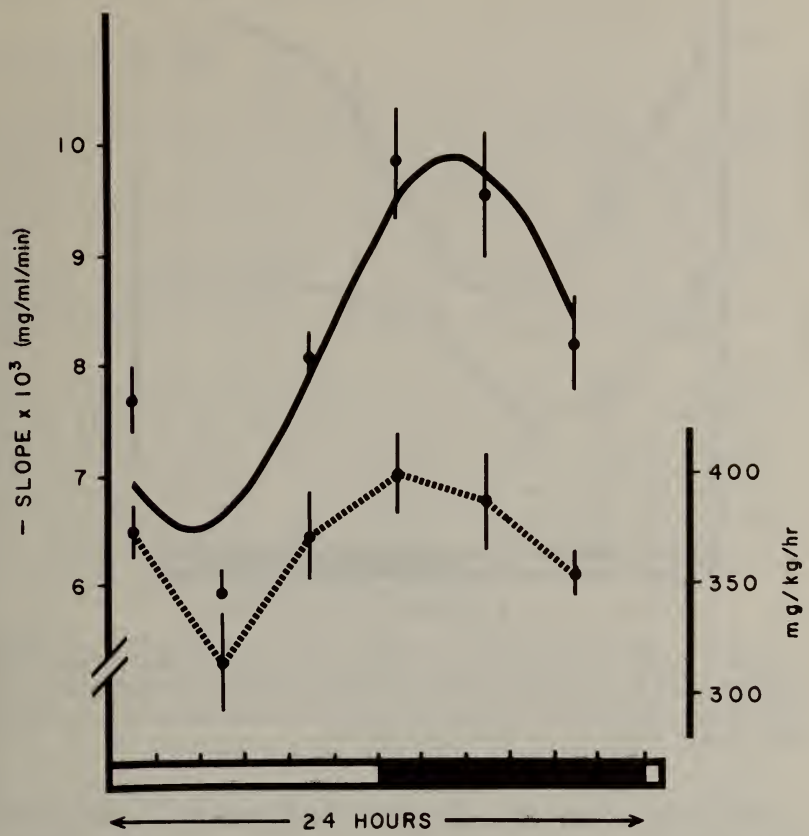

FIGURE 3.--Chronovariation in blood-ethanol clearance rates ±SE superimposed by the best fitting cosinor-derived 24-hour curve (solid line) and ethanol metabolic rate ±SE (dashed line) in virgin female rats (N = 8) per data point

value. These data are expressed as milligrams ethanol/killigram/hour and are shown by the dashed line. Although the metabolism data did not allow a successful fit of a 24-hour cosine curve, the Student's t-test showed a significant difference (p = .02) between the minimal rate near the midlight phase and the maximal rate early in the dark phase of the LD cycle.

After a 1-week recovery period, these rats were placed with 2-month-old males at the midpoint of the dark phase of the LD cycle. The males were removed, and vaginal smears were taken at the beginning of the next light phase. This mating procedure continued daily until sperm were found in the smear, indicating

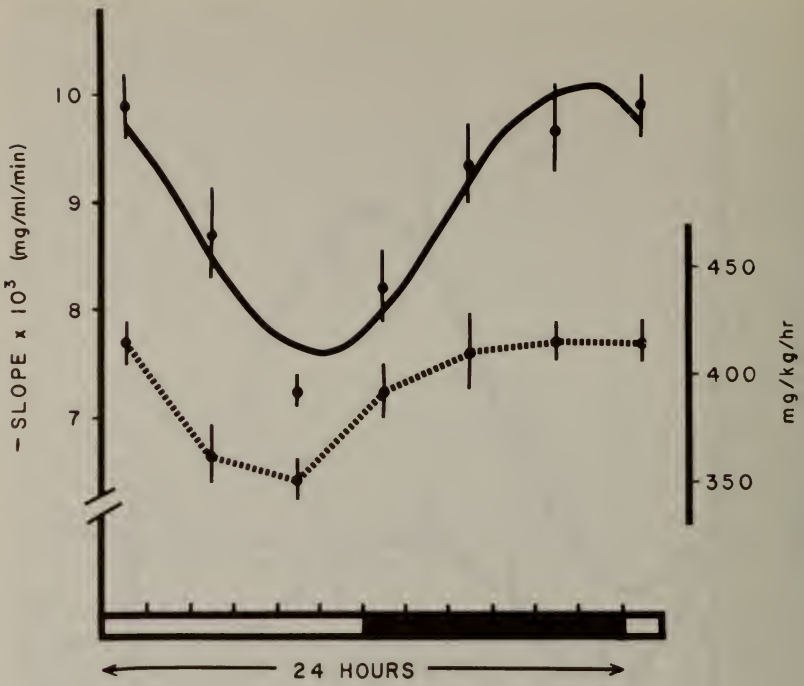

FIGURE 4.--Chronovariation in blood-ethanol clearance rates ±SE superimposed by the best fitting cosinor-derived 24-hour curve (solid line) and ethanol metabolic rate ±SE (dashed line) in pregnant female rats (N = 8 per data point)

a presumptive pregnancy. On gestation day 12 or 13, the female rats were again injected with the same dose of ethanol at the same clock hour as in the preceding (virgin) study. Similar sampling and analytical procedures were followed. Figure 4 shows the circadian variation in blood-ethanol clearance rates with the best fitting cosine curve (solid line) and the rate of ethanol metabolism (dashed line) for the second study. The time of maximal metabolic rate, which was phase delayed approximately 6.3 hours from that of the prepregnant study, corresponds to the time of maximal clearance rate previously found for male rats (Sturtevant and Garber 1980, 1981; Sturtevant et al. 1981a). The data suggest that there may be a greater degree of colony synchronization during pregnancy than during the virgin state. The vaginal smears taken daily during mating procedures indicated that the individual estrus cycles were not in synchrony. The increased percent rhythm (86 percent for the virgin rats, 91 percent for the pregnant animals) adds further evidence that the pregnancy-induced hormonal changes had a synchronizing effect on both the group clearance rate and the metabolic rate rhythms. Although the mesor (mean rhythm value) was slightly

increased during pregnancy, the rhythm amplitudes of the two studies do not differ significantly.

MECHANISMS

A preliminary study of possible mechanisms underlying the rhythm in ethanol metabolic rate began with an investigation of circadian fluctuations in activity levels of hepatic alcohol dehydrogenase (ADH) and the microsomal ethanol-oxidizing system (MEOS) of enzymes. Three groups of 24 male rats each were maintained on a 12 hour:12 hour LD cycle. Rat chow and drinking fluid were continuously available. For one group (the chronic group), the sole drinking fluid was 15 percent ethanol in tap water. The two remaining groups (acute and control groups) had tap water to drink. Fluid consumption was recorded daily.

At 22 weeks after the beginning of the study, each of the three groups was divided into four subgroups ($N = 6$) to determine enzyme levels at four equidistant intervals over the LD cycle (1 hour after the onset of the light and the dark phases and the two midpoints between). The acute group was injected with 1.5 g/kg ethanol 3.5 hours before sacrifice, and blood-ethanol clearance rates were determined as before. Blood-ethanol levels of the chronic group were measured immediately before sacrifice. The control group remained ethanol naive.

Following decapitation, livers were quickly harvested, rinsed, and weighed, and a 1-g sample was homogenized in 9 ml 0.15M KCl. ADH activity levels were determined spectrophotometrically by using the supernatant fraction obtained by centrifugation at 9,000g for 30 minutes at 4°C, according to the methods of Anderson et al. (1979). An aliquot of this supernatant fraction was then centrifuged at 100,000g for 1 hour. The resultant microsomal pellet was washed and resuspended, and the activity level of MEOS was determined by the method of Lieber and DeCarli (1972). Protein levels were measured by the method of Lowry et al. (1951). All assays were performed in duplicate.

Figure 5 shows ADH levels assessed as micromoles of substrate oxidized per minute per milligram protein. In all three groups, peak levels were noted at 03:00, with minimal levels at 15:00 hours. Although enzyme levels in the acute and control groups did not differ from each other, at each time point they were decreased by 22 to 55 percent in the chronic group. Similar curves were found when ADH levels were calculated per minute per gram liver or per kilogram rat.

MEOS activity levels, assessed as micromoles of acetaldehyde formed per minute per milligram protein, are shown in figure 6. The low levels seen in both the control and acute groups showed no significant change over the 24-hour span. In contrast, MEOS activity levels in the chronic group were approximately 500 percent greater at 15:00 than at 03:00 hours. It is apparent that

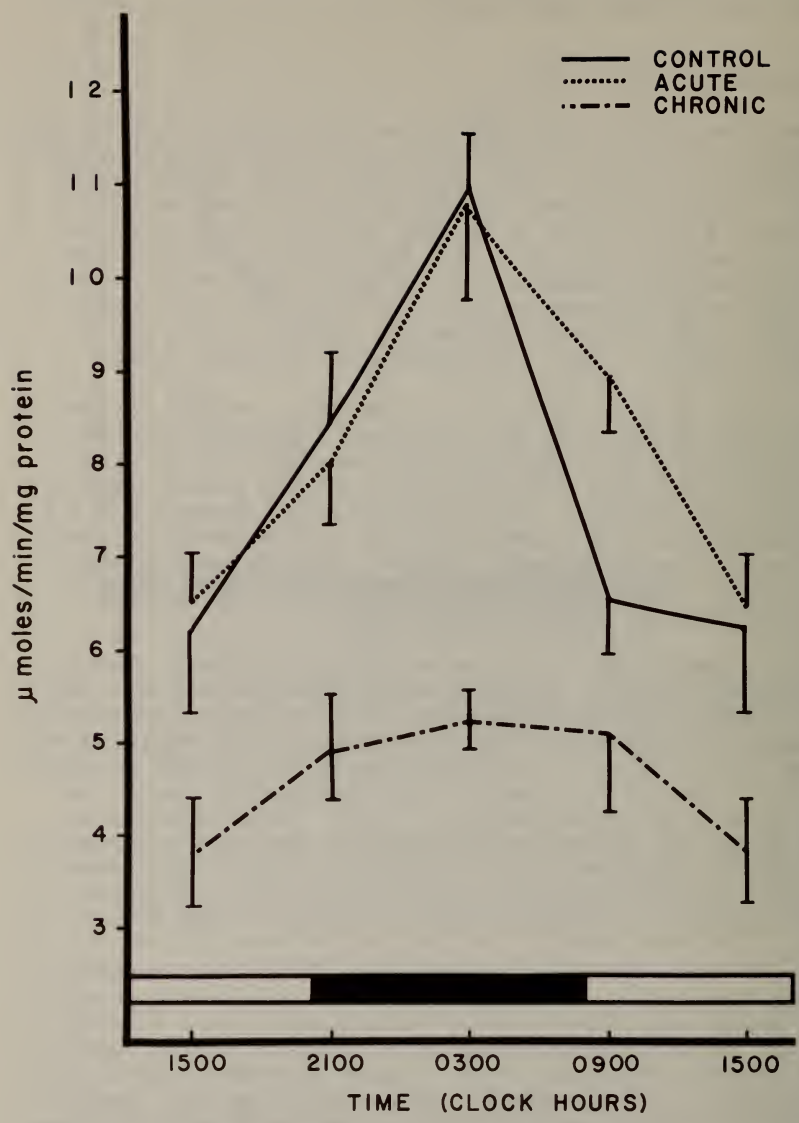

FIGURE 5.--Hepatic ADH levels ±SE in three groups of male rats ($\underline{N}$ = 24) after 22 weeks of continuous ethanol availability to the chronic group. Acute group was singly dosed (1.5 g/kg, IP) 3.5 hours before sacrifice. Control group was ethanol naive. One-quarter of each group was sacrificed every 6 hours. Horizontal bar shows LD regimen. From Sturtevant, R.P., and Garber, S.L. Circadian variation in alcohol dehydrogenase activity in rats, in press. Reproduced with permission, copyright S. Karger AG, Basel.

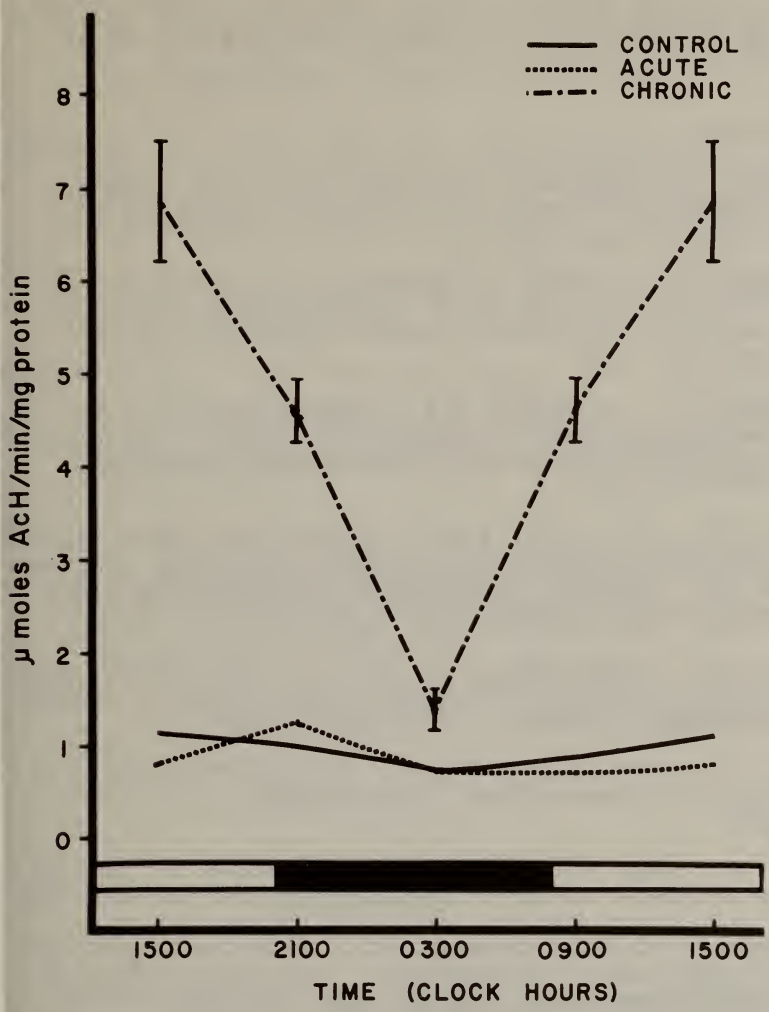

FIGURE 6.--Hepatic MEOS activity levels ±SE in three groups of male rats. Details of figure as in figure 5.

the circadian rhythms of the two enzyme systems were 180° out of phase with each other. In the ethanol-naive (control) and singly dosed (acute) groups, ADH peak activity levels fell near the circadian phase of maximal metabolic rate. The chronically dosed rats showed suppressed ADH levels, but MEOS levels varied in a circadian fashion. Clearly, long-term continuous exposure to ethanol has altered not only the metabolic processes by which hepatic degradation of ethanol has occurred but also the circadian timing of the reactions. An understanding of the

events that underlie these metabolic alterations and their effects on other ethanol-induced physiological changes awaits further investigation.

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Effect of Hormones on Ethanol Metabolism

Esteban Mezey

The ingestion of alcohol has been associated with various hormonal changes, and hormones, in turn, have recently been found to affect the activity of ethanol-metabolizing enzymes and ethanol metabolism. Ethanol is eliminated from the body mainly by metabolism in the liver and only minimally by urinary excretion, pulmonary alveolar air, and perspiration. The principal enzyme responsible for the oxidation of ethanol is hepatic alcohol dehydrogenase (ADH). Ethanol is also oxidized to a lesser extent by a microsomal ethanol oxidizing system (MEOS) (Lieber and DeCarli 1972). Changes in the rate of ethanol elimination depend not only on the activity of the enzymes but on the availability of coenzymes. Various studies have shown that the rate of ethanol metabolism that is catalyzed by ADH depends principally on the mitochondrial reoxidation of NADH (nicotinamide adenine dinucleotide phosphate) generated during ethanol oxidation (Meijer et al. 1975). Liver ADH has a broad substrate specificity. Besides ethanol it oxidizes a variety of physiological steroidal and nonsteroidal substrates (Li 1977). Hence, an effect of hormones on liver ADH activity may affect endogenous metabolism of physiological substrates in unknown ways. Also hormones that have an effect on ADH may be substrates for the enzyme in the liver or other organs.

STRESS AND CATECHOLAMINES

Stress is well known to activate the hypothalamic-hypophyseal-adrenocortical axis and the sympathetic nervous system (McCann et al. 1971). Stress induced by exposing rats to cold for various days resulted in increases in both the rate of ethanol elimination in vivo (Forbes and Duncan 1961; Platonow and Coldwell 1966, 1968; Videla et al. 1975) and in the rate of ethanol oxidation by liver slices in vitro (Videla et al. 1975). Stress induced by immobilization of rats for 2.5 hours/day for 7, 14, and 42 days resulted in increases in liver ADH activity (Mezey et al. 1979). A single immobilization had no effect on the enzyme activity. A maximum increase of 83 percent in the activity of the enzyme

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was obtained after 14 days of stress, and this was associated with a doubling of the rate of ethanol elimination. The rate was increased from 332.2±29.5 (SE) mg/kg body weight/hour in the immobilized rats as compared with a control value of 153.9±17.1 mg/kg body weight/hour ($p < .001$). The increase in the rate of ethanol elimination was not associated with any changes in the activity of MEOS, microsomal catalase, cytochrome P-450, and NADPH cytochrome c reductase. A decrease in the hepatic phosphorylation potential ($[ATP]/[ADP][P_i]$) due to a decrease in [ATP] and an increase in $[P_i]$ was found; however, there were no changes in either oxygen consumption by liver slices or in hepatic (Na^+-K^+)-stimulated ATPase. Hence, increased liver ADH activity rather than increased mitochondrial oxidative rate appeared to be the cause of the increased ethanol elimination after stress induced by immobilization.

Chronic administration of epinephrine for 3 weeks increased the rate of ethanol elimination in male rats (Petermann et al. 1979). This effect was associated with an increased oxygen consumption by liver slices, suggesting that the increased ethanol elimination was a result of an increased mitochondrial oxidative rate; however, the activity of liver ADH was not determined. Beta adrenergic blockade did not alter the effect of epinephrine in increasing the rate of ethanol elimination.

CORTICOSTEROIDS

The administration of hydrocortisone, corticosterone, or corticotropin for 6.5 days to male rats had no effect on liver ADH activity (Mezey and Potter 1979). Similarly the administration of corticosterone for 2 weeks to mice had no effect on the enzyme activity (Sze 1975). Also adrenalectomy had no effect on the enzyme activity in either rats (Mezey and Potter 1979; Raiha et al. 1967) or mice (Sze 1975). In one study, a 10 to 15 percent reduction in liver ADH activity was found in rats 6 days after adrenalectomy (Fazekas and Rengei 1968), but there was no indication that this change was statistically significant. On the other hand, the presence of an intact adrenal appears to be necessary to obtain increases in liver ADH produced by various stimuli. Adrenalectomy prevented increases in liver ADH produced by chronic administration of ethanol in male mice (Sze 1975) and by experimentally induced uremia in male rats (Mezey and Potter 1978).

GROWTH HORMONE AND HYPOPHYSECTOMY

Stress and uremia, which increased liver ADH activity in male rats, are associated with increases in plasma growth hormone (Mezey and Potter 1979), and decreases in plasma testosterone (Repcekova and Mikulaj 1977). Administration of growth hormone to normal male rats resulted in a small but significant increase

in liver ADH activity (Mezey and Potter 1979). Surprisingly, hypophysectomy resulted in a marked twofold increase in the enzyme activity, which was suppressed by the administration of growth hormone. These results indicate that liver ADH activity is affected by growth hormone and by other unknown factors that are altered by hypophysectomy. More recently, castration (Rachamin et al. 1980; Mezey et al. 1980; Cicero et al. 1980) and thyroidectomy (Mezey and Potter 1981) were found to increase liver ADH activity, which was suppressed by the administration of testosterone and triiodothyronine, respectively.

ANDROGENIC STEROIDS

Higher activities of liver ADH and of rates of ethanol elimination have been found in various strains of female rats (Buttner 1965; Erikkson and Malstrom 1967; Rachamin et al. 1980) and mice (Erikkson and Pikkarainen 1968; Collins et al. 1975) and mice (Erikkson and Pikkarainen 1968; Collins et al. 1975) as compared with males. Castration prevented the normal decreases in liver ADH activity that occur in spontaneously hypertensive rats of 5 to 13 weeks of age (Rachamin et al. 1980), and it increased liver ADH activity in postpubertal Sprague-Dawley rats (Mezey and Potter 1979; Cicero et al. 1980). The increase in liver ADH was suppressed by the administration of testosterone (Rachamin et al. 1980; Mezey and Potter 1979; Cicero et al. 1980). By contrast, administration of testosterone to intact rats resulted in a small paradoxical increase in the enzyme activity in one study (Mezey and Potter 1979) but in no changes in other studies (Rachamin et al. 1980; Cicero et al. 1980). No changes in the Michaelis-Menten constants for ethanol or NAD^+ or in the isoenzyme pattern on starch gel electrophoresis were found in the enzyme after castration or the administration of testosterone. The increased activity of the enzyme was accompanied by an increased rate of elimination in the castrated animals, suggesting that under these circumstances, as was the case after stress, ADH is rate limiting. However, a much greater increase in total heptic ADH activity (98 percent) than in the rate of ethanol elimination (50 percent) and a greater decrease in the cytosolic free $NAD^+/NADH$ ratio observed in the castrated than in the control animals suggested that the reoxidation of NADH can in turn become rate limiting at high oxidation rates, resulting from the increased liver ADH activity (Mezey et al. 1980).

Liver ADH and the rates of ethanol elimination were also increased after chronic morphine administration to rats, which lowered serum testosterone levels to 15 percent of control values (Cicero et al. 1980). Testosterone's effect in suppressing the enzyme activity could be caused by a direct inhibitory effect of testosterone or its metabolites, or it could be caused by an effect on the synthesis or degradation of the enzyme. In one study the enhanced enzyme activity in the castrated animals returned to control levels in 16 to 24 hours after injection of a single dose of testosterone (Cicero et al. 1980). This delay in the in vivo inhibition of the

enzyme suggested that a metabolite of testosterone, such as dihydrotestosterone, may be the inhibitory compound. Dihydrotestosterone, which is a more potent androgen than testosterone in bioassays in the rat (Wilson 1972), was found to be a substrate of liver ADH in the reductive direction and a competitive inhibitor of ethanol oxidation by the enzyme (Mezey and Potter 1982). The administration of a single dose of dihydrotestosterone inhibited liver ADH 4 hours after injection, but this effect was not present at later intervals after injection. This transient in vivo inhibition of liver ADH was associated with a delay in ethanol elimination. Testosterone had no effect on the enzyme activity in vitro or after its administration in vivo; however, it resulted in a slight delay in ethanol elimination. MEOS was found to play no role in the changes in ethanol elimination observed after castration and dihydrotestosterone administration since its activity remained unchanged. Dihydrotestosterone concentrations that produced inhibition were much higher than those found circulating in normal animals. Hence, removal of a direct inhibitory effect of dihydrotestosterone is an unlikely mechanism for the observed increases in liver ADH and ethanol elimination after castration. Effects of androgenic steroids on synthesis or degradation of the enzyme are more likely potential mechanisms.

ADH activity has a wide tissue distribution, and the effects of testosterone or other hormones described for the liver enzyme may be different for the enzyme in other tissues. The activity of kidney ADH is much higher in the kidneys of male than of female mice (Ohno et al. 1970; Sabolic and Dembic 1981). Castration decreases the activity in the kidneys of male mice, and testosterone administration increases the activity in the kidneys of castrated and intact male and female mice.

ESTROGENS

Ovariectomy has no effect on ADH activity or on rates of ethanol elimination in female rats (Rachamin et al. 1980; Mezey et al. 1981). The administration of estradiol also was without effect in spontaneously hypertensive intact or ovariectomized female rats (Rachamin et al. 1980), but it slightly increased the activity of the enzyme but not the rate of elimination in intact female Sprague-Dawley rats (Mezey et al. 1981). By contrast, administration of estradiol to intact spontaneously hypertensive male rats resulted in decreases in both the enzyme activity and the rates of ethanol elimination (Rachamin et al. 1980). Further, estradiol administration prevented the inhibitory effects of testosterone in castrated male rats. Administration of ethynyl estradiol increases cytoplasmic aldehyde dehydrogenase activity in intact and castrated male rats and inhibits the high K_m mitochondrial aldehyde dehydrogenase in castrated but not intact rats (Messiha et al. 1980).

THYROID HORMONES

Thyroidectomy (Mezey and Potter 1981) and the chronic administration of propylthiouracil (Hillbom and Pikkarainen 1970) result in increases in liver ADH activity. The enzyme in the thyroidectomized animals has similar Michaelis-Menten constants for ethanol and NAD^+ than the enzyme from control animals. In initial studies three isoenzymes of ADH activity were demonstrated on thyroidectomized animals on starch gel electrophoresis, as compared with two in control animals (Mezey and Potter 1981); however, more recently three isoenzymes were also detected in control animals (Mezey unpublished observations). The administration of triiodothyronine decreased the enzyme activity in control animals and suppressed the enhanced activity in thyroidectomized animals. Triiodothyronine inhibited ADH in vitro in both the control and thyroidectomized animals. The inhibition was competitive with respect to NAD^+ and was uncompetitive with respect to ethanol. Previous studies with horse liver ADH suggested that triiodothyronine interfered with coenzyme binding by blocking the binding site of the ADP-ribose portion of the coenzyme (Gilleland and Shore 1969).

Thyroidectomy results in decreases in the pituitary and serum contents of growth hormone (Mezey and Potter 1981) and in decreases in serum testosterone (Bruni et al 1975); thus, it could have an effect on liver ADH by these indirect mechanisms. These possibilities, however, were excluded by showing that administration of growth hormone had no effect on the enzyme activity in thyroidectomized animals (Mezey and Potter 1981) and that thyroidectomy resulted in increases in enzyme activity in female rats, which were similar in magnitude to those found in male rats (Mezey, unpublished observations).

The activity of the hepatic MEOS was not affected by thyroidectomy or by the administration of triiodothyronine to male rats (Mezey and Potter 1981). However, in another study administration of either thyroxine or triiodothyronine increased the MEOS activity in female rats (Moreno et al. 1979).

Thyroidectomy did not result in any changes in the rate of ethanol elimination. The factor that most likely limits an increase in ethanol oxidation by ADH in vivo in the thyroidectomized animal is a defect in the transport of reducing equivalents into the mitochondria. This is suggested by a decrease in the cytosol but not the mitochondrial-free $NAD^+/NADH$ ratio after ethanol administration in the thyroidectomized animals as compared with the parallel decrease in both ratios in the control animals. The defect in transport of reducing equivalents is most likely related to decreases in α-glycerophosphate dehydrogenase and hence the α-glycerophosphate shuttle after thyroidectomy (Lee and Lardy 1965). In addition, decreases in mitochondrial function and oxygen consumption demonstrated in thyroidectomized animals (Tata et al. 1962) suggest that the reoxidation of NADH by the respiratory chain may also be decreased.

Increases in ethanol metabolism have been observed in hyperthyroid patients (Ugarte and Peresa 1978) and after the administration of low doses of thyroxine in rats (Israel et al. 1975b). The increased ethanol metabolism in rats occurred in association with increased oxygen consumption in liver slices and despite a slight decrease in liver ADH activity. These changes are consistent with a principal dependence of ethanol elimination on the mitochondrial reoxidation of reducing equivalents.

ADAPTIVE INCREASE IN ETHANOL ELIMINATION

Chronic ethanol administration results in increases in ethanol metabolism rates (Tobon and Mezey 1971) and decreases in serum testosterone levels (Cicero and Badger 1977; Rachamin et al. 1980). Hence, it has been suggested that these increases may be due to a decrease in testosterone concentration. This hypothesis is supported by the observation that the increase in liver ADH activity and in the rate of ethanol metabolism obtained in spontaneously hypertensive rats after chronic ethanol administration was markedly inhibited by castration (Rachamin et al. 1980). It is also possible that the increase in ethanol metabolism is mediated by an effect of ethanol on the hypothalamic-pituitary-gonadal axis. Decreases in serum testosterone after chronic ethanol ingestion are associated with no or only small increases in plasma gonadotropins (Gordon et al. 1976; Van Thiel and Lester 1976). Further, plasma LH (luteinizing hormone) and testosterone decrease in parallel after acute ethanol administration (Cicero et al. 1979), and ethanol administered to castrated animals markedly suppresses the expected increase in serum LH concentration (Cicero et al. 1978). LH depression was readily overcome by the injection of LH-releasing hormone, suggesting an ethanol-induced hypothalamic dysfunction. Hypophysectomy, as mentioned before, results in an increase in liver ADH activity, but it is not known if this is associated with an increase in ethanol elimination. However, the hypothesis that links levels of testosterone or gonadotropins with ADH activity and ethanol elimination is tenuous in most strains of rats, where increases in ethanol elimination after chronic ethanol feeding are not accompanied by changes in liver ADH (Tobon and Mezey 1971; Lieber and DeCarli 1972). Instead increases in hepatic (Na^+-K^+)-stimulated ATPase and in hepatic oxygen consumption after chronic ethanol feeding (Israel et al. 1975a) suggest that the increased rate of ethanol elimination after chronic ethanol feeding is the result of increased mitochondrial oxidative capacity. Thyroid hormones may play a prominent role in the increased ethanol metabolism since propylthiouracil preferentially decreases the enhanced rate of ethanol metabolism in animals after chronic ethanol consumption (Britton and Israel 1980).

SUMMARY AND CONCLUSIONS

The activity of liver ADH and rates of ethanol metabolism are affected by various hormones. Stress, which stimulates the hypothalamic-hypophyseal-adrenocortical axis and the sympathetic nervous system but decreases circulating testosterone levels, increases the activity of liver ADH and rates of ethanol metabolism. Hypophysectomy, castration, and thyroidectomy each result in increased liver ADH activity, which is suppressed by growth hormone, testosterone, and thyroid hormones, respectively. The suppressant effect of androgenic steroids and of thyroid hormones may be partly due to the demonstrated direct inhibitory effects of these hormones on the activity of the enzyme. However, alterations in enzyme turnover are probably the principal mechanism for the observed changes in enzyme activity produced by hormones. Increased enzyme activity after castration is associated with an increased rate of ethanol elimination, suggesting that in this circumstance the activity of liver ADH is a limiting factor in ethanol elimination. In contrast, thyroidectomy failed to increase ethanol elimination, and this is most likely related to decreases in the transport of reducing equivalents into mitochondria and in mitochondrial functions that occur in thyroidectomized animals.

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Role of Thyroid Hormones in the Swift Increase in Alcohol Metabolism (SIAM)

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INTRODUCTION

Numerous studies have documented the fact that chronic treatment with ethanol increases the rate of ethanol elimination in humans (Mendelson and Mello 1966; Ugarte et al. 1977; Misra et al. 1971), experimental animals (Hawkins et al. 1966; Wendell and Thurman 1979; Videla et al. 1973; Mezey 1976), and a variety of in vitro preparations of liver (Videla et al. 1973; Thurman et al. 1976). Recently, Yuki and Thurman (1980b) showed that after a large dose of ethanol, this adaptive increase was observed in a few hours, both in vivo in the rat (Bleyman and Thurman 1979) and in the perfused liver--i.e., the swift increase in alcohol metabolism (SIAM). Ethanol uptake by the perfused liver was nearly doubled 2.5 hours after ethanol pretreatment in vivo (5.0 g/kg). This phenomenon was associated with a marked elevation in hepatic oxygen uptake, which could be partly accounted for by depressed rates of glycolytic ATP (adenosine triphosphate) production (Yuki and Thurman 1980a).

Using liver slices, Israel et al. (1975) showed that the increases in ethanol uptake and oxygen consumption following chronic treatment with ethanol could be blocked by treatment with the antithyroid drug, 6-n-propyl-2-thiouracil. Hypothyroidism and propylthiouracil (PTU) treatment are known to increase glycogen reserves in the liver, which might lead to an increase in the glycolytic rate (Bargoni et al. 1966; Chilson and Sacks 1959). The purpose of these studies, therefore, was to examine the effect of PTU on SIAM, oxygen uptake, and rates of glycolysis in the perfused rat liver. The data indicate that PTU pretreatment indeed prevents the SIAM effect, although its action is not mediated by an effect on glycolytic flux.

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METHODS

ANIMALS

Female albino rats (Sprague-Dawley, 80 to 150 g) received laboratory chow and water ad lib. Animals in the ethanol-pretreated group received one single dose of 10 percent ethanol orally (5.0 g/kg) 2.5 hours before surgery. Controls were intubated with corn oil. PTU (50 mg/kg daily in corn oil) was administered by gastric intubation for 10 days. A hypothyroid state was evidenced by marked enlargement in the thyroid glands.

NONRECIRCULATING LIVER PERFUSION

The nonrecirculating liver-perfusion technique has been described (Scholz et al. 1973). Krebs-Henseleit (Krebs and Henseleit 1932) bicarbonate buffer (pH 7.4), saturated with oxygen (95 percent) and carbon dioxide (5 percent), was pumped into the liver via a cannula placed in the vena cava and flowed past an oxygen electrode before it was discarded. Ethanol was infused into the influent perfusate. The oxygen concentration in the effluent perfusate was determined continuously with a polytetrafluoroethylene- (Teflon) shielded platinum electrode. Metabolic rates were calculated from influent-effluent concentration differences and the constant flow rate and were expressed per gram wet weight of the liver.

ANALYTICAL

Samples of effluent perfusate were collected every 2 minutes and analyzed for glucose, lactate, pyruvate, and ethanol by standard enzymatic procedures (Bergmeyer 1970).

RESULTS

Experiments were done on livers from four groups of animals: (1) controls, (2) ethanol (2.5 hours) pretreated, (3) PTU-pretreated rats, and (4) ethanol plus PTU-pretreated rats. After 20 minutes of preperfusion, samples of effluent perfusate were collected every 2 minutes for the enzymatic determination of rates of glucose production and rates of glycolysis (lactate plus pyruvate production). In the period from 20 to 40 minutes of perfusion, rates of glucose production and rates of glycolysis ranged from 70 to 90 and from 70 to 80 μmol/g/hour, respectively During the same period, oxygen uptake was of the order of 100 μmol/g/hour. At 40 minutes of perfusion, ethanol (final concentration = 1.5m$\underline{M}$) was infused. Ethanol uptake by livers from normal rats was about 80 μmol/g/hour during the period between 40 and 60 minutes of perfusion. Ethanol infusion under these conditions reduced the rate of glycolysis and caused a slight but detectable stimulation of oxygen uptake. Rates of glucose production were unaffected by the infusion of ethanol.

The rates of ethanol uptake by livers from the four groups of rats studies are compared in table 1. Rates of ethanol uptake were diminished slightly by PTU pretreatment. In vivo treatment of rats with ethanol increased the rates of ethanol uptake by the perfused livers from 84 to 132 μmol/g/hour in livers from normal animals; however, in livers from PTU-pretreated rats, this increase was not observed.

Livers from PTU-pretreated rats consumed oxygen at rates that were about two-thirds those of the normal untreated animals (table 2). Following in vivo pretreatment with ethanol, the oxygen uptake of the liver in the absence of ethanol in the perfusate was doubled (table 2). In contrast, ethanol pretreatment had little if any effect on oxygen uptake in livers from PTU-pretreated rats--i.e., the stimulation of oxygen uptake by the in vivo ethanol treatment was blocked by PTU.

Lactate plus pyruvate production is a good index of the rate of glycolysis since pyruvate concentrations are below the K_m of pyruvate dehydrogenase under these conditions (Thurman and Scholz 1977). During the 20- to 40-minute perfusion period, rates of glycolysis were between 70 and 80 μmol/g/hour in livers from the normal rats (table 3). In livers from PTU-pretreated, ethanol-pretreated, and PTU plus ethanol-pretreated rats, basal rates of glycolysis were much lower (ca. between 28 and 39 μmol/g/hour).

TABLE 1.--Ethanol uptake by perfused rat liver*

Pretreatment in vivo	Ethanol uptake (μmol/g/hour)
None	83.9±2.4
PTU	61.1±1.7
Ethanol	132.4±2.5
PTU plus ethanol	54.6±1.1

*Rates of ethanol uptake were measured in perfused (Klingman and Goodall 1957) rat livers during an infusion of ethanol (1.5m*M* final concentration) for 20 minutes after stabilization of hepatic oxygen uptake. Samples of effluent perfusate were measured at 2-minute intervals and were analyzed for ethanol enzymatically. Female Sprague-Dawley rats were treated for 10 days with PTU (50 mg/kg). Ethanol (5 g/kg) was administered via gastric intubation 2.5 hours before perfusion. Normal rats were untreated. Results are mean ±SEM for all values measured in each treatment group (*N* = 4 to 6).

TABLE 2.--Oxygen uptake by perfused rat liver*

Pretreatment in vivo	Basal oxygen uptake (μmol/g/hour)
None	98.2±2.4
PTU	64.4±0.7†
Ethanol	213.8±0.6†
PTU plus ethanol	81.2±1.1

*Basal rates were measured for 10 minutes following stabilization of hepatic oxygen uptake. Oxygen uptake was monitored continuously with a polytetrafluoroethylene-shielded platinum electrode placed in the effluent perfusate. The influent oxygen concentration was maintained by an oxygenator and calibrated from the arterio-venous concentration differences, the flow rate, and the liver wet weight. Results are mean ±SEM for all values measured in each treatment group (N = 4 to 6).

†$p < .001$.

TABLE 3.--Glycolysis in perfused rat liver*

Pretreatment in vivo	Basal lactate plus pyruvate production (μmol/g/hour)
Normal	75.4±1.5
PTU	39.6±1.3†
Ethanol	28.6±1.0†
PTU plus ethanol	28.6±2.6†

*Glycolysis was measured by summation of lactate and pyruvate production (see table 1). Results are mean ±SEM for all values measured in each treatment group (N = 4 to 6).

†$p < .001$.

TABLE 4.--Effects of fructose on oxygen and ethanol uptake in perfused livers from normal and PTU-pretreated rats*

Pretreatment in vivo	Addition	Uptake (percent of control) Oxygen	Ethanol
None	None	100±6	100±12
None	Fructose (4m*M*)	126±3	164±4†
PTU	None	62±1†	30±4†
PTU	Fructose (4m*M*)	65±1†	35±2†

*Conditions were as in tables 1 and 2. Ethanol (1.5m*M* final concentration) infusion was begun at 20 minutes of perfusion and represents the control condition for this experiment. At 40 minutes, fructose (4m*M*) was infused. Data are represented as percent of control of data from values similar to those shown in tables 1 to 4 (*N* = 4).

†$p < .05$.

Following the addition of ethanol to the perfused liver, marked inhibition in glycolytic flux was observed in livers from normal and PTU-pretreated rats (table 3); however, ethanol addition to the perfusate had no effect on the rates of glycolysis in liver from ethanol-pretreated or ethanol plus PTU-pretreated rats.

Fructose stimulates oxygen uptake by increasing the supply of ADP (adenosine diphosphate) to the mitochondrial respiratory chain subsequent to its phosphorylation by the highly active ketohexokinase (Scholz and Nohl 1976). It is by this mechanism that fructose stimulates hepatic ethanol uptake. Since PTU pretreatment decreased glycolysis (table 4), an ADP-consuming process, and also decreased oxygen uptake (table 2), the effect of fructose was examined. As expected, fructose increased oxygen and ethanol uptake in livers from normal rats (table 4); however, it had no effect on either variable in livers from PTU-pretreated rats (table 4).

DISCUSSION

Chronic ethanol administration has been reported to increase the rates of oxygen consumption and ethanol metabolism in the livers of experimental animals (Hawkins et al. 1966; Wendell and Thurman 1979; Videla et al. 1973; Mezey 1976; Thurman et al. 1976). Recent studies (Yuki and Thuman 1980a) have shown that this

effect can also be obtained in 2 to 3 hours after the administration of one large dose of ethanol (the SIAM effect). A hypermetabolic state induced by either of these techniques depends on adrenergic mechanisms since adrenergic blocking agents (Yuki and Thurman 1980a; Bernstein et al. 1975) and adrenalectomy block this phenomenon (Bernstein et al. 1975; Yuki and Thurman 1980a; Videla 1978). A hypermetabolic state following chronic ethanol treatment has also been shown to depend on thyroid status. Thyroidectomy and PTU pretreatment have been reported to markedly reduce or block the increase in oxygen uptake and reduce or block the increase in oxygen uptake and ethanol metabolism produced by chronic ethanol treatment (Israel et al. 1975). Present findings show that PTU treatment also can prevent the effect of ethanol on both oxygen consumption and ethanol metabolism in the hypermetabolic state induced by acute addition of ethanol (tables 1 and 2). Therefore, thyroid hormones are necessary for ethanol to stimulate ethanol and oxygen uptake, whether it is administered acutely or chronically.

It is known that there is a direct relationship under most circumstances between the rate of oxygen consumption and the rate of ethanol metabolism (Videla et al. 1973; Thurman et al. 1976) because the respiratory chain is responsible for the oxidation of NADH (nicotinamide adenine dinucleotide) produced by alcohol and acetaldehyde dehydrogenase reactions. However, not all investigators have observed an increase in oxygen uptake after ethanol treatment (Schaffer et al. 1980). This relationship between oxygen consumption and ethanol metabolism was also observed in the four experimental conditions used in the present studies ($\bar{r}$ = 0.86). For example, PTU pretreatment prevented most of the elevation of oxygen uptake due to ethanol pretreatment in vivo in perfused livers both in the presence and absence of ethanol in the perfusate (table 2). Thus, by preventing a more rapid oxidation of reducing equivalents at the mitochondrial level, PTU may also prevent an increase in the rate of ethanol metabolism. It is unlikely that PTU reduces the rate of ethanol metabolism by decreasing alcohol dehydrogenase (ADH) activity; the activity of this enzyme is actually increased after PTU treatment (Hillbom 1971; Israel et al., unpublished observations).

By its well-documented antithyroid actions, PTU affects the rates of several intracellular ATP-utilizing processes (Ismail-Beigi and Edelman 1970). It is via this mechanism that PTU affects oxygen uptake by the liver. We have proposed previously that both the chronically induced hypermetabolic state and the SIAM effect increase ATP-utilizing reactions, although the exact nature of the ATP-consuming reactions is still not clear (Thurman et al. 1976; Yuki and Thurman 1980a; Bernstein et al. 1973; Yuki et al. 1980).

EFFECT OF ETHANOL ADDED IN VITRO

We have shown previously (Thurman and Scholz 1977) that addition of ethanol to the perfused liver of fed rats leads to a small (5 to 10 percent) but reproducible increase in the rate of oxygen uptake (table 2). This effect is due to inhibition of glycolysis at glyceraldehyde-3-phosphate dehydrogenase due to elevation in the $NADH/NAD^+$ redox state as a consequence of ethanol metabolism (Furfine et al. 1969). Since glycolysis is an ATP-producing reaction, this inhibition by ethanol leads to a greater rate of supply of ADP to the respiratory chain, thus increasing oxygen uptake. This effect is only evidenced in the presence of adequate glycogen stores (e.g., in the fed state). In these studies, with the exception of the PTU-pretreated rat, a good correlation was observed between the reduction in lactate plus pyruvate production and the increase in oxygen uptake following addition of ethanol to the perfusate (tables 2 and 3).

EFFECT OF SIAM AND PTU ON GLYCOLYSIS

One possible mechanism for the increase in oxygen consumption in the SIAM state is a reduction in cellular glycogen stores, thus resulting in lower rates of glycolysis (Yuki and Thurman 1980a). Since it is well known that glycogen levels increase after PTU, we predicted that after PTU treatment the increase in oxygen uptake due to ethanol treatment would be diminished.

Large doses of ethanol cause the release of epinephrine and norepinephrine (Klingman and Goodall 1957). These hormones elevate blood glucose and deplete intrahepatic glycogen stores (Yuki and Thurman 1980a). Therefore, substrate depletion in all likelihood accounts for the lower rates of glycolysis observed in the SIAM animals (table 3).

It was our expectation that since hypothyroidism elevated glycogen stores, an increased oxygen uptake after ethanol treatment would be reversed by PTU by making substrate available for glycolysis. An increase in substrate availability occurred since rates of glucose production remained high in perfused livers of PTU- or PTU-plus-ethanol-pretreated rats, and PTU pretreatment prevented the inhibition of glucose production induced by acute treatment with ethanol. However, PTU abolished the increased rate of oxygen consumption following the acute ethanol treatment without preventing the reduction in glycolysis. This is most likely due to the fact that PTU treatment per se leads to a reduction in energy demands. PTU is known to reduce the phosphorylation potential (Hassinen 1971), which in turn reduces glycolysis (Atkinson 1966). Thus, PTU does not prevent the elevation in oxygen consumption produced by chronic ethanol treatment by affecting glycolysis.

If rates of glycolysis are decreased by PTU treatment, why isn't oxygen uptake stimulated? This is a crucial question since Yuki

and Thurman (1980a) have postulated a role of diminished glycolysis in the mechanism of SIAM. Some insight into this question came from experiments with fructose. Fructose, like decreased glycolysis, increased the supply of ADP subsequent to its phosphorylation via the highly active ketohexokinase (Scholz and Nohl 1976). As expected, fructose stimulated ethanol and oxygen uptake in livers from normal rats (table 4). In contrast, fructose did not stimulate oxygen uptake in livers from PTU-pretreated rats (table 4). If we assume that fructose phosphorylation occurs in livers from PTU-pretreated rats, then PTU treatment has somehow prevented ADP stimulation of hepatic respiration. If this hypothesis is correct, one would not expect decreased glycolysis (table 3) to stimulate respiration in livers from PTU-pretreated rats.

EFFECT OF ACUTE ETHANOL ADMINISTRATION AND TREATMENT WITH PTU ON GLUCOSE PRODUCTION

PTU pretreattment probably prevented the effect of adrenergic hormones released by acute ethanol treatment on the glycogen phosphorylase system (Harrison 1964). We have postulated previously that hypoxia due to a faster utilization of oxygen by the liver can result in zone 3 and lead to alcoholic hepatitis and liver necrosis (Israel et al. 1975; Orrego et al. 1979). Recently, Ji et al. (in press) demonstrated that alcohol treatment increases the oxygen gradient between the periportal and pericentral regions of the liver lobule. PTU administration to patients with alcoholic hepatitis has resulted in a faster improvement in their condition (Orrego et al. 1979). Further, PTU has been shown to minimize hepatic necrosis in animals that were subjected to low oxygen tensions after both acute and chronic alcohol administration (Israel et al. 1975; Britton et al. 1979). The protective effects of this drug on liver cell necrosis may be related to the effect of PTU on oxygen consumption observed in this study.

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ROLE OF HORMONES IN FUNCTIONAL TOLERANCE TO AND PHYSICAL DEPENDENCE ON ETHANOL

Floyd E. Bloom, Chairperson
George F. Koob
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John R. Wenger
Jatinda M. Khanna

Overview

Floyd E. Bloom

In focusing on the effects of alcohol on experimental animals or humans, it is observed that with repeated exposure, the dose-response relationship for the effects has "shifted to the right." This means that to produce a given effect, a bigger dose is needed as the exposure is repeated. This rightward shift is the basic phenomenon of tolerance.

Tolerance can be detected on the effects of alcohol on membranes and enzymes of several organ systems, such as liver, muscle, and kidney. The brain can address the functions of these same organs in two general ways: directly, by virtue of the innervation of these organs, and indirectly, through the hypothalamic-hypophyseal axis and peripherally circulating hormones. Some elements of the reduced effectiveness of alcohol on the brain can be "learned." Further, Barry's chapter explicitly shows that the environment in which an animal or human receives alcohol can contribute associational clues that are necessary for the tolerance to appear. When control administrations are given in this environment, the tolerant animal's response reveals the opposing adaptational mechanisms by which the behavioral tolerance to alcohol is apparently generated.

However, the actual molecular mechanisms that underlie the development of these opposing adaptational responses to the behavioral actions of alcohol are not known with certainty. What has been reported is that a common set of endocrine hormones may influence both processes--namely, the process of "learning" and the process of "environment-dependent" tolerance to alcohol. In this section, these possible relationships are probed further.

Koob examines the hypotheses and evidence that pertain to the ability of the endocrine hormones, arginine vasopressin (AVP) and corticotropin releasing factor (CRF), to influence certain modes of responding in experimental animals; this report leads to the view that the actions of AVP per se are aversive when this hormone is given in doses that have been reported to influence behavioral performance after subcutaneous administration, and that this aversive action may produce sufficient arousal to enhance the effectiveness of the conditional stimuli that regulate the animal's behavior. CRF produces a more direct arousal that may also facilitate learning in some situations.

Hoffman examines the role that AVP and its analogs may play in what she and Tabakoff refer to as "environment-independent" tolerance to alcohol. Melchior examines the hormonal associations involved in her studies of "environment-dependent" tolerance to alcohol in mice. Wenger reports further data on the interactions between AVP (or CRF) administration to rats (in doses known to be effective in standard aversive learning paradigms) and alcohol, administered chronically in environmentally cued conditions. Khanna reports on the interactions between vasopressin analogs that are said to be devoid of peripheral vascular actions and the central serotonin projections, and concludes that the central monoamine system is necessary for the effects of lysine vasopressin on extinction of tolerance to alcohol.

Although these data and their interpretations are far from harmonious, and in fact at this stage seem quite contrary, the conservative view may be that we simply lack adequate information to fit together these disparate observations. A great deal of work is still needed. The interested reader should await these observations before making firm conclusions.

Finally, of what use to the organism is "tolerance"? Certainly, from a strict pharmacological viewpoint, tolerance to the behavioral effects of ethanol would appear to be antiadaptational since the existence of tolerance in a voluntary consumer requires that person to consume greater doses to achieve the desired effect and thereby exposes all the organ systems to increasing opportunities for the toxic effects of alcohol. Were there no such tolerance, the effects could be obtained with a constant, and smaller, dose, and the risks of toxicity would be somewhat reduced.

What, then, might be usefully served by the phenomenon of tolerance and its underlying cellular and molecular mediators? At the cellular and molecular levels of research on neurotransmission, recent research has clearly demonstrated the modifications in responsiveness to specific transmitters when animals are chronically exposed to drugs that stimulate or antagonize the effects of that transmitter. Constant exposure to agonist analogs lowers responsiveness to the transmitter, while chronic treatments with antagonists heighten responsiveness. It is as though the healthy nervous system alters responsiveness in a direction that will allow the transmission of information to become normalized despite the chronic presence of the drug that causes the initial perturbations in transmission. Perhaps this cellular regulation of interneuronal communication leads to an apparent blunting of the effects of the perturbing agent, which we then recognize as tolerance to that agent.

If we also recognize that the normal action of the endocrine system is to maintain internal homeostasis, we may come to reflect on the behavioral actions of endocrine hormones as revealing their normal homeostatic functions, rather than a specific additional role in activating the cellular mechanisms of either

"tolerance" or "learning." I will not be surprised if we eventually come to recognize that the so-called "learning" effects of certain peptides reflect more on the adaptive significance of tolerance than vice versa.

Interaction of Vasopressin and Corticortropin Releasing Factor With Stress: Implications for Alcohol Tolerance Research

George F. Koob

THE STRESS RESPONSE

The general concepts of stress theory have undergone little modification and refinement over the past 40 years, and most investigators would still define stress as the response of the pituitary adrenal system to a variety of physical and psychological stimuli. These stimuli are conveyed, via humoral or neural pathways, to the anterior pituitary, which responds by secreting ACTH. ACTH secretion is, in turn, controlled primarily by one or more corticotropin releasing factors (CRF) secreted by cells in the hypothalamus (Vale et al. 1981). ACTH stimulates the adrenal cortex to secrete glucocorticoids, which have widespread effects on metabolism, such as glucogenogenesis, hyperinsulinemia, lysis of lymphoid tissue, increased gastric secretion, and reduced inflammatory and antibody responses. The glucocorticoids decrease ACTH production by a negative feedback on the hypothalamus and pituitary. These physiological changes in response to increased hypothalamic-pituitary action are accompanied by changes in behavior. For example, ACTH injections can reverse avoidance deficits associated with hypophysectomy, and in normal animals ACTH facilitates avoidance learning and delays habituation to aversive stimuli, actions generally interpreted as increases in attention or alertness (Sandman et al. 1975).

It is also generally accepted that "emotional" stimuli will cause a release of posterior pituitary hormones, such as arginine vasopressin (AVP) and oxytocin (Pickford 1969). Stimuli that can induce the release of AVP range from severe hemorrhage and water deprivation to emotional stress in dogs (O'Connor and Verney 1942) and shock in rats (Thompson and De Wied 1973: van Wimersma Greidanus et al. 1979).

Efforts to identify the antecedent physiological mechanisms of the hypothalamic-pituitary "stress responses" have commonly defined two categories of stress: systemic stress and neurogenic

stress. Systemic stress is stress that activates ACTH release by a direct hormonal action on the pituitary, presumably via the median eminence; neurogenic stress is stress that activates ACTH release by an indirect action on the pituitary via neural connections. This distinction is based on the observation that complete or partial hypothalamic deafferentiation or localized hypothalamic lesion can abolish or reduce the adrenocortical response to photic, acoustic, olfactory, and peripheral nerve stimulation, but such lesions do not alter the adrenocortical response to systemic stresses, such as ether stress or injection of epinephrine (Conforti and Feldman 1976). Indeed, it is through these central nervous system (CNS) pathways to the hypothalamus that presumably the adrenocortical response is triggered by psychological factors, and they may also be the basis for the autonomic arousal associated with anxiety. However, there is one alternative means by which behavioral or physiological responses to stress or anxiety might be mediated by the hypothalamic-pituitary system in an organism--that is, via direct neurotropic action of a peptide such as CRF in the CNS itself. Thus, just as pathways project to the hypothalamus from the limbic areas to activate the pituitary-adrenal axis, so might peptide-containing neurons project back to these same areas to mediate appropriate behavioral responses to stress.

Evidence for both types of interactions can be seen in our recent work that examined the behavioral effects of vasopressin and CRF. Both peptides clearly produce behavioral effects, particularly in stressful situations or in learning situations that are motivated by aversive events. Our preliminary studies in search of their mechanisms and site of action follow.

VASOPRESSIN AND BEHAVIORAL ACTIONS

PROLONGATION OF EXTINCTION IN AN AVERSIVELY MOTIVATED TASK

In early work De Wied found that hypophysectomized rats are deficient in a number of behavioral situations, especially the acquisition and extinction of aversively motivated tasks. These deficiencies were reversible by administration of a crude pituitary extract, pitressin, and in later work, by AVP (the natural vasopressin of all mammals except swine) in microgram amounts injected subcutaneously (SC). Vasopressin also reversed the behavioral deficits observed in Brattleboro strain rats with congenital diabetes insipidus due to their inability to synthesize vasopressin (for review see Koob and Bloom 1982).

Although attempts to replicate these effects in other laboratories have met with only mixed success, our recent studies (Koob et al. 1981; Le Moal et al. 1981) on active avoidance have successfully reproduced and extended some of the findings of De Wied and associates. AVP injected both SC and intracerebroventricularly (ICV) delayed the extinction of an active avoidance response.

However, a pressor antagonist analog of AVP (1-deaminopenicillamine, 2-(o-methyl)tyrosine AVP) (dPTyr(Me)AVP), which prevented the pressor response, also abolished the effects of SC-injected AVP on prolongation of extinction of active avoidance (figure 1). This blockade may indicate either that signals from peripheral visceral sources play an important role in the subsequent behavioral changes, or that the receptors at which AVP elicits its pressor effect are similar to those that lead to its behavioral action.

Some evidence, with different synthetic analogs of AVP, suggests that vasopressin produces its behavioral effects independently of classic renal or pressor effects. For example, [des-9-glycinamide, 8-lysine] vasopressin (DGLVP), a vasopressin with

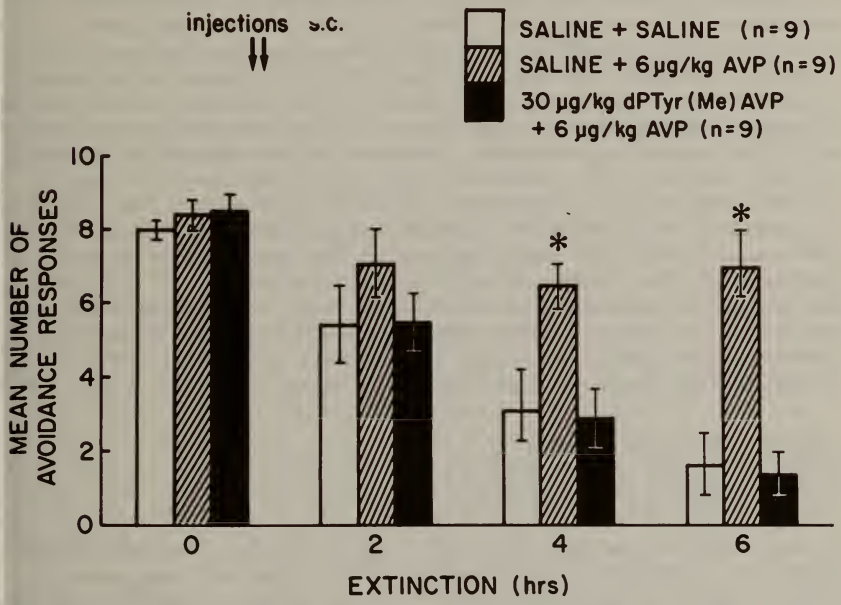

FIGURE 1.--Effects of AVP and AVP plus dPTyr(Me)AVP on extinction of active avoidance behavior. After 3 days of training, rats that met the criteria of at least 7 successful avoidances during the first 10 trials of extinction were injected (SC) with saline (twice at 2.0-minute intervals), AVP plus saline or AVP plus dPTyr(Me)AVP immediately after the first set of 10 extinction trials on day 4. Rats that received AVP alone show persistent avoidance throughout the 6 hours of observation, when tested on 10 trials at each of the three next 2-hour intervals. Rats that received either saline with no peptide or both peptides extinguished this active avoidance behavior at similar rates. Asterisk: significantly different from both the saline with no peptide and both peptides groups; $p < .05$ (Newman-Keuls test following analysis of variance). From Le Moal et al. 1981. Reprinted by permission from Nature, 291:491. Copyright 1981 by Macmillan Journals Limited.

supposedly minimal physiological activity, can have similar effects on avoidance learning as vasopressin (Walter et al. 1975), and, along with a host of other compounds, it can reverse experimentally induced amnesia (Rigter et al. 1974). Also central ICV injections of vasopressin can produce similar behavioral effects of doses that are 1,000 times less than those used after peripheral injections (De Wied 1976; Koob et al. 1981). Based on these observations some investigators believe that the hypothalamic-fugal (nonendocrine) AVP circuits represent the basis for direct actions on the neural mechanisms involved in memory processing (e.g., for the acquisition, consolidation, and retrieval of acquired information). However, the behavioral effects of AVP in learning/memory paradigms could also arise as a consequence of the endocrine actions, since the resulting arousal can significantly improve the acquisition and subsequent performance of conditioned responses. The endocrine actions of AVP might, therefore, act as an arousing stimulus, which then secondarily enhances performance in various conditioned paradigms (perhaps by increasing attentiveness to environmental cues). For example, since the successful animal studies have almost exclusively used aversively motivated learning paradigms, perhaps the endocrine properties of AVP contribute to produce an even stronger negative conditioned stimulus (i.e., increases in blood pressure). This might be expected to increase the animals' motivation to continue performing, in much the same manner as turning up the current intensity of the electric shock during acquisition. AVP might, therefore, produce a behavioral result that is consistent with the notion of memory improvement without actually affecting "memory processes" (i.e., the presumptive molecular events by which memory is stored and retrieved) at all.

UNCONDITIONED AVERSIVE PROPERTIES OF PERIPHERALLY ADMINISTERED VASOPRESSIN

If experimentally administered AVP were aversive, the behavioral effects of peripherally administered AVP might be independent of any memory-enhancing action, and the apparent memory actions of AVP could be explained without reference to any direct effects on memory substrates, e.g., aroused rats may "simply learn better." We have recently used two behavioral assays to examine possible aversive properties of AVP: (1) a conditioned taste-aversion paradigm, and (2) a conditioned place paradigm (Ettenberg et al. 1983).

For the conditioned taste-aversion experiment, rats were deprived of water and given 0.1 percent saccharin to drink for 20 minutes. Then half of the rats were injected with a moderate dose of AVP, and the remaining rats (controls) were injected with saline. Treatments were given on days 1 and 3; no treatments were provided on even-numbered days when only water was available again for 20 minutes each day. On day 5 the rats were presented with two drinking bottles. One contained the saccharin solution

and the other water. This two-bottle free-choice (preference) test lasted 20 minutes, and then the bottles were removed. Twenty-four hours later the procedures for days 7 to 10 were repeated in the manner described for days 1 to 5. In this test, we observed a significant conditioned aversion to saccharin in AVP-treated rats but not in vehicle-treated rats (Ettenberg et al., unpublished data). This effect indicates convincingly some aversive consequences as a result of AVP administration.

For the conditioned place-preference test, rats were tested for avoidance or preference of a distinctive environment previously paired with the putative aversive substance, in this case AVP. The experiment consisted of two phases: (1) a conditioning phase that lasted 8 consecutive days and (2) a test phase on the ninth day. Conditioning involved the injection of the animal with either AVP or vehicle and placing it, for 30 minutes each day, in one of two types of large plastic (Plexiglas) boxes. One box was constructed of black plastic, including the floor, which was wiped with a 2 percent solution of acetic acid. The other box was the same size, but it was made of white plastic, had a floor of soft animal bedding, and no acetic acid odor.

During the conditioning phase the rats (six per group) received either 1.2 μg/kg or 6.0 μg/kg AVP. This procedure continued for 8 days so that each rat had experienced four AVP injections paired with one of the two types of boxes, and four vehicle injections paired with the other type of box. On day 9, animals were tested for 10 minutes in a large rectangular box that was designed to offer the rat the choice between a side of black plastic wiped with acetic acid and a side of white plastic with animal bedding on the floor. Either aversive or reinforcing effects of AVP can be detected by the test, depending on whether the subjects subsequently avoid or prefer the side associated with AVP injections. In this study the rats showed significant avoidance of the side associated with AVP, again indicating that these peripheral doses are aversive.

CONCLUSIONS

One can tentatively conclude from the literature and from our results that information about a learning situation is apparently consolidated more effectively in rats treated with AVP than in rats treated with vehicle. However, we also found that these effects of peripherally administered AVP can be reversed by a pressor antagonist analog of vasopressin, and that vasopressin itself can produce aversive unconditioned effects. Thus, although AVP may also operate synaptically within the brain as released from neural sources, it is not clear that peripheral administration communicates directly with these presumptive receptors. In fact, one would have to assume not only that AVP gets to these central receptors from the periphery but that AVP also operates directly on a memory mechanism. However, we feel that one should not presume a direct action on memory mechanisms of this peripheral source of AVP as long as alternative explanations

remain. For example, the present demonstration that AVP has aversive, and probably, arousing, properties makes it premature to conclude that AVP acts directly on memory mechanisms. One might intuitively expect that the enormous increases in blood pressure produced by behaviorally effective systemic doses of AVP would be aversive and, therefore, arousing. When this source of arousal is prevented by pretreating the subjects with a potent AVP pressor antagonist, the potentiation of learned performance is blocked. We hypothesize that it is the alerting or arousing consequences produced by administering a mildy aversive agent that may be responsible for improvements in the performance of animals in various memory/learning paradigms. How central AVP stores interact with this peripheral arousal mechanism is under investigation.

CRF AND BEHAVIORAL ACTIONS

This dichotomy between the peripheral and central actions of a hypothalamic peptide is even better exemplified by our recent work with the 41-amino acid peptide, corticotropin releasing factor (CRF). CRF is presumably the long-sought hypothalamic neurohumor, with the specific function of releasing ACTH from the anterior pituitary. Thus, it may be considered the final common pathway for the neurohumoral control of ACTH and its biological actions.

As mentioned, the physiological changes in response to increased ACTH release are thought to be paralleled by alterations in behavior that have been associated with increases in alertness and attention (De Wied 1977). For example, ACTH has been shown to delay extinction of active avoidance behavior, passive avoidance behavior, and food-motivated behavior. In many reports, effects on concentration, visual attention, and motivation have been observed following treatment with ACTH 4-10 (Sandman et al. 1975; De Wied 1977). Other investigators have noted that ACTH has anxiogenic effects in rats (File 1979).

LOCOMOTOR ACTIVATION PRODUCED BY CRF (ICV)

However, preliminary histological evidence suggested that terminals containing immunoreactive CRF are located in pontine and diencephalic brain (Bloom et al. 1982) and led to the possibility that CRF might have a direct neurotropic in the central nervous system. Indeed, recent work in our laboratories has demonstrated that CRF injected ICV in rats produces a dose-dependent locomotor activation in rats that cannot be reproduced by peripheral injection. See figure 2. Besides increases in normal locomotion and sniffing, the rats injected with CRF (1.5 to 150 picomoles) exhibited some unique behavior that appeared to reflect a general behavioral activation, such as a bizarre elevated walking and rhythmic walking forward and backward (Sutton et al. 1982).

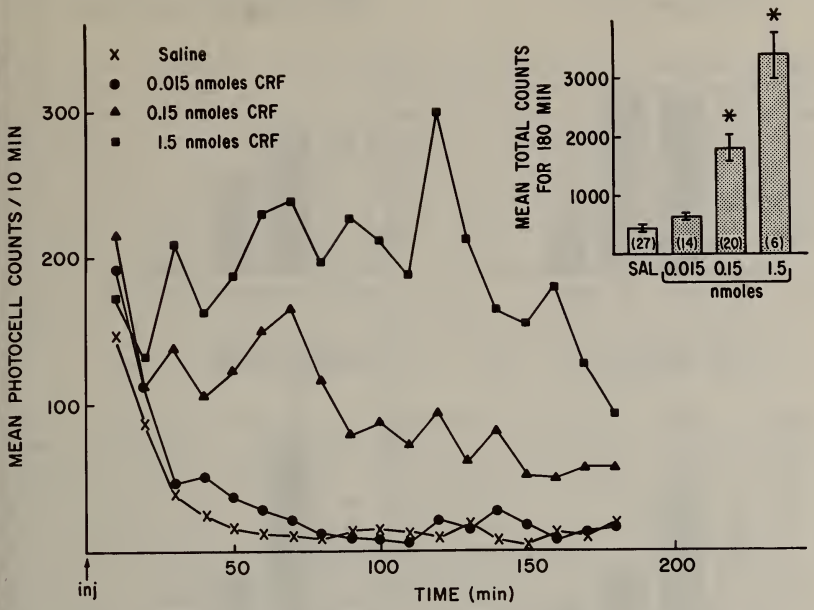

FIGURE 2.--Locomotor response after ICV infusions of 0.1, 1, and 10 μg, corresponding to 0.015, 0.15, and 1.5 nmol of CRF, respectively. Ordinate refers to total photocell counts for each 10-minute period of a 3-hour test. Asterisk: significantly different from saline control and from each other; $p < .05$ (Neuman-Keuls test following analysis of variance). Mean total counts for 180 minutes represent average ±SEM. From Sutton et al. 1982. Reprinted by permission from Nature, 297:332. Copyright 1982 by Macmillan Journals Limited.

Although other peptides, such as the endorphins and ACTH, have been shown to produce increases in spontaneous behavioral activity, the nature of the CRF response differed substantially from the responses observed with these other peptides (Dunn et al. 1979; Segal et al. 1979). CRF does not produce the initial depressant phase followed by bursts of locomotor activity that characterizes ICV injections of opioid peptides; nor does CRF produce the "stretching-yawning syndrome" observed after ICV injection of ACTH. In contrast, the activation of CRF at the lower doses appeared to be an exaggeration of the normal activation produced by introducing the animal into a familiar environment.

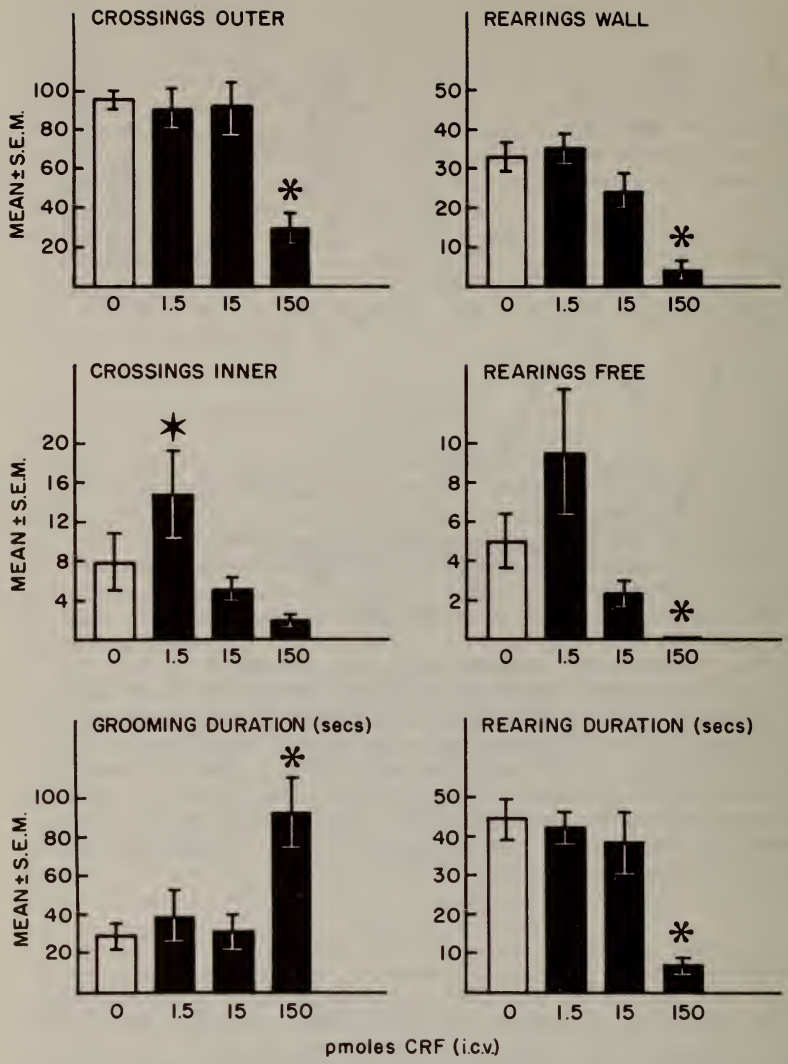

FIGURE 3.--Effects of CRF (ICV) on behavior of nondeprived rats in the open field. Values are mean ±SEM. Asterisk: significant difference compared with saline-injected animals (Neuman-Keuls test). A posteriori tests were made only after obtaining significant differences using analysis of variance (ANOVA). F_{max} $p < .05$: raw data for inner crossings and free rearing were square-root transformed for ANOVA. Asterisk: significantly different from 150 pmol. Adapted from table 1 of Sutton et al. 1982. Data reproduced by permission from Nature, 297:332. Copyright 1982 by Macmillan Journals Limited.

CRF INDUCTION OF STRESS-RELATED BEHAVIOR

Rats were tested in a novel open field after ICV injection of similar doses of CRF (1.5 to 150 picomoles) and showed responses that were consistent with an increased emotional state or increased sensitivity to the stressful aspects of the situation. Here the rats showed decreases in locomotion and rearing (see figure 2). Typically, a rat injected with 150 picomoles of CRF and placed 60 minutes later in the open field moved hesitantly to the outer squares and then either circled the open field, remaining close to the floor, or remained in one of the corners grooming or hesitantly moving forward and backward. In contrast, saline-injected animals rapidly circled the open field, rearing and exploring, and eventually made forays into the inner squares of the center of the open field (Sutton et al. 1982; see figure 3).

Similar results were obtained in another open-field test that has been validated as sensitive measure of "anxiety" (Britton and Britton 1981). Rats that were deprived of food for 24 hours were placed in a novel circular open field. The open field was highly illuminated, where a single food pellet weighing about 6.0 g was secured to a pedestal in the center of the field. The number of approaches to the food pedestal, the number of bouts of grooming and rearing, and the total amount of food eaten were recorded, and the mean amount of food eaten per approach was then calculated for each animal (Britton et al. 1982; see figure 4).

Rats injected ICV with 0, 1.5, 15, and 150 picomoles of CRF showed a dose-dependent decrease in the amount of food eaten in this test (see figure 3). Rearing and approaches to food were also decreased, and grooming was increased as observed in the open-field study with nondeprived rats.

CONCLUSIONS

These preliminary results suggest that CRF acts centrally to produce a dose-dependent behavioral activation in a familiar environment where control rats rapidly habituate and go to sleep. In contrast, in a more stressful, novel environment, rats show behavior that is consistent with an increase in the stress of the situation. These results are consistent with the hypothesis that CRF in the brain may play a behavioral role that is consonant with its hypothesized function in the classical endocrine response to stress. Indeed, the profile of the effects of these higher doses of CRF is the opposite of that which appears with administration of anxiolytic drugs (Britton et al. 1982).

Of future interest will be the determination of whether this activation can facilitate learning and, if so, under what conditions. Equally intriguing will be the pursuit of interactions among circulating steroids, endorphins, and vasopressin and the physiological and behavioral actions of CRF.

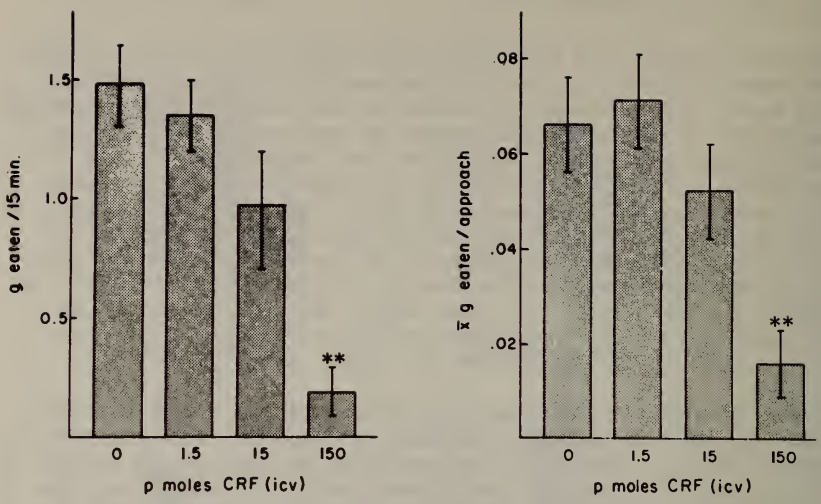

FIGURE 4.--Effects of CRF on the behavior of fasted rats tested for 15 minutes in the open field. Left: total amount eaten during the 15-minute test. Right: total amount eaten divided by the number of approaches to the food pedestal (mean g/approach). Values are the mean ±SEM. Asterisks: significantly different from saline control (CRF); $p < .01$ (Mann Whitney-U test). From Britton et al. 1982. Reprinted with permission from Life Sciences, 31:366. Copyright 1982 by Pergamon Press.

IMPLICATIONS FOR TOLERANCE RESEARCH

NONSPECIFIC INTERACTIONS

We have outlined some reliable effects of peptides on behavior that, at least for vasopressin, have been hypothesized to result from an action on a "memory/learning" substrate. In our pursuit of the mechanism and site of action for these effects, several facts became obvious. First, from the literature virtually all vasopressin effects have been obtained on aversively motivated tasks. Second, a potent pressor antagonist of AVP can reverse the behavioral effects of systemic administration of AVP. Third, AVP itself can have potent aversive and behaviorally disruptive effects acutely. Thus, we have hypothesized that these peripheral "arousal" effects may account for the behavioral effects observed with systemic administration. Until such nonspecific arousing-type effects can be eliminated from the repertoire of actions of peptides that influence behavior, it is unnecessary to postulate peptides acting as memory hormones.

For CRF similar caveats hold but from the other side of the blood-brain barrier. CRF (ICV) clearly induces behaviorally arousing actions, and it may influence performance in a variety of other behavioral situations. Indeed, as with the case of peripherally administered AVP, CRF (ICV) can augment the "stressfulness" of novel and presumably stressful environments.

TOLERANCE/LEARNING/STRESS

Much evidence now exists to support the hypothesis that there is a significant learning component in the development of tolerance to the behavioral effects of a drug such as alcohol. This learning was originally described as behaviorally augmented tolerance, where rats acquired tolerance more rapidly if they were allowed to "practice" the task in question under the influence of the drug (Kalant et al. 1971). Recent work has suggested that learning is more important than previously thought; here only rats that were allowed to practice a treadmill task under the influence of alcohol became tolerant; control animals that received alcohol after the test failed to show any behavioral tolerance (Wenger et al. 1981).

Because learning has an important role in the development of tolerance and because moderate increases in arousal can significantly improve the acquisition and subsequent performance of conditioned responses (Hebb 1966), it is not difficult to imagine how vasopressin or CRF could alter the development of tolerance. For AVP, this might be a peripheral activation; for CRF, perhaps it is a central activation. Thus, optimal learning, and perhaps optimal development of tolerance, will correspond to an optimal level of arousal, and pushing this function too far may result in a deterioration in learning due to performance problems. Particularly intriguing for future research, if this hypothesis if correct, will be the importance of such variables as dose, motivation, and the difficulty of the task.

Future work may also profitably concentrate on the means by which the neuroendocrine and visceral regulatory axes of the brain monitor the results of executive commands to secrete pituitary and peripheral hormones for maintenance of the internal milieu, and this work should examine whether even "nonendocrine" metabolites of active peptides can function as feedback signals. This type of information may help address the larger question of what the functional value of tolerance is: molecular, cellular, or behavioral. Perhaps tolerance is the attempt of the body to monitor the normal signals of the environment despite the interference of the drug treatment, and the hypothalamic-pituitary system may play a key role in this phenomenon as it does in homeostasis in general.

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Modulation of Ethanol Tolerance by Neurohypophyseal Hormones

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ETHANOL EFFECTS ON VASOPRESSIN RELEASE

Acute and chronic exposure to ethanol affects the function of several endocrine systems, leading to alterations in the plasma levels or actions of various hormones (e.g., Van Thiel 1982). One might expect that these changes in hormonal activity would, in turn, contribute to the organism's response to ethanol. The effects of ethanol on the release of antidiuretic hormone, [8-arginine]vasopressin (AVP), from the mammalian posterior pituitary have been known for some time. This peptide hormone is synthesized in neurons of the hypothalamus, carried by axonal transport to the posterior pituitary, and released into the circulation in response to changes in plasma osmolality or extracellular fluid volume. AVP acts on the kidney to increase water retention and, thus, restore plasma osmolality and volume to normal levels. Acute exposure to ethanol inhibits AVP secretion during the time that blood-ethanol levels are rising. At the time of peak blood-ethanol levels, AVP secretion has been reported to be increased over basal levels, although this "rebound" may depend on the age of the subjects (Helderman et al. 1978). During chronic treatment of rats with ethanol, plasma AVP levels were reported to be increased above control values. However, the chronically treated rats still responded to an acute administration of ethanol with a decrease in circulating AVP (see Hoffman and Tabakoff 1981). The mechanism by which ethanol affects AVP secretion is not totally clear but seems to be due to a central action, and it may be related to ethanol's modulation of the activity of neurotransmitter systems that normally influence AVP release (e.g., cholinergic and noradrenergic systems).

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AVP AND MEMORY

The fact that ethanol alters the release of AVP provides one rationale for studying the influence of this hormone on the response to ethanol. A second reason lies in the "extra-endocrine" effects of AVP. The hormone appears to influence processes related to memory or learning, as measured by its ability to modulate extinction of active and passive avoidance behaviors (van Ree et al. 1978), and to attenuate amnesia caused by various agents (see Walter et al. 1980). Not all investigators accept these results, but it is generally agreed that under controlled conditions, vasopressin can enhance memory consolidation and/or retrieval or expression of information, in animals and perhaps in humans (Weingartner et al. 1981).

Various experimental manipulations that interfere with memory consolidation also interfere with the development or expression of tolerance to ethanol (LeBlanc and Cappell 1976). Such findings led to the hypothesis that memory, or learning, and tolerance, which both represent central nervous system (CNS) adaptive processes, may share certain underlying mechanisms. It seemed reasonable, therefore, that AVP might also modulate ethanol tolerance.

AVP AND ETHANOL TOLERANCE

Our initial experiments were designed to evaluate the effect of AVP on the maintenance of ethanol tolerance. This design was chosen to provide some analogy to studies in which vasopressin was shown to maintain (inhibit the extinction of) avoidance behavior (van Ree et al. 1978). In our experiments, C57B1 mice were administered ethanol in a liquid diet for a length of time such that functional (and not metabolic) tolerance to the hypnotic, hypothermic, and discoordinating effects of ethanol developed. Following withdrawal from ethanol and after overt withdrawal signs had dissipated, the animals were tested for tolerance, and control and ethanol-treated groups were each subdivided into groups that received once-daily injections of saline or AVP (10 μg/mouse, ca. 400 nmol/kg). Although all ethanol-exposed animals initially displayed tolerance to the hypnotic and hypothermic effects of ethanol, this tolerance dissipated over 3 to 6 days after ethanol withdrawal in the saline-treated mice. In contrast, tolerance was maintained as long as AVP was administered (9 to 12 days after withdrawal) in the ethanol-exposed, AVP-treated mice (see Hoffman and Tabakoff 1981). After termination of the peptide treatment, tolerance dissipated at a rate similar to that observed in the saline-treated mice.

Oxytocin, the other neurohypophyseal hormone, did not influence the rate of tolerance dissipation. Peptide treatment did not alter ethanol metabolism, and animals in which AVP maintained tolerance regained the righting reflex at higher brain-alcohol levels than

nontolerant animals, indicating that AVP prolonged functional tolerance to ethanol (Hoffman et al. 1980; Hoffman and Tabakoff 1981).

We recently found that AVP also prolongs the duration of tolerance to low-dose, discoordinating effects of ethanol, as measured on the accelerating Rotarod. These findings, in conjunction with our previous results indicating that acute influences of AVP on the response to ethanol did not interfere with determinations of tolerance (Hoffman et al. 1980), support the contention that AVP modulates tolerance per se and does not simply affect the tests used to assess tolerance.

AVP AND PHYSICAL DEPENDENCE ON ETHANOL

Most of the theories that have been proposed to explain the development of tolerance to and physical dependence on drugs have been predicated on the concept that tolerance and physical dependence both arise simultaneously from adaptation to a drug-induced perturbation of CNS function. However, with respect to ethanol, neurochemical evidence suggests that tolerance and physical dependence are dissociable phenomena (Tabakoff and Ritzmann 1977). In certain of our experiments, AVP was administered to mice throughout the period of exposure to ethanol as well as after withdrawal. We did not observe any change in withdrawal severity in these mice, although AVP did prolong tolerance (see Hoffman and Tabakoff 1981). Thus, our results are consistent with the idea that tolerance can be affected independently of physical dependence. On the other hand, Crabbe and Rigter (1980) reported that an analog of vasopressin, [des-9-glycinamide]-AVP (DGAVP), increased the severity and duration of ethanol-withdrawal symptoms (i.e., seizures). In their experiments, DGAVP was administered <u>during</u> withdrawal; in our experiments, AVP was not given on the day of withdrawal. These investigators showed that DGAVP did not change the seizure threshold to pentylenetetrazole in naive animals, and they concluded that enhancement of withdrawal-seizure intensity was not due to a proconvulsive effect of DGAVP but was actually a modulation of physical dependence. However, AVP has been reported to promote a "kindling"-like process in rats (Kasting et al. 1980) and the repeated injections of DGAVP during withdrawal may have caused subliminal changes that affected the intensity or duration of ethanol-withdrawal seizures. Thus, DGAVP may well affect withdrawal symptomatology without altering the mechanisms that underlie physical dependence.

FORMS OF ETHANOL TOLERANCE

The finding that AVP can prolong the duration of functional tolerance to ethanol raises several important questions. First, one of the incentives for studying the effects of AVP on tolerance was the reported influence of this hormone on memory-related

processes. The fact that AVP does "inhibit the extinction" of ethanol tolerance, in analogy to its effects on memory, is in line with the idea that similar adaptive mechanisms may be involved in tolerance and memory. Recent studies, however, indicate that ethanol tolerance is a more complex phenomenon than was previously recognized (see Tabakoff et al. 1982). In addition to these distinctions, it has recently been proposed that ethanol tolerance may be classified as "environment-dependent" or "environment-independent" (Tabakoff et al. 1982; Melchior et al. "Influencing Environment-Dependent Tolerance to Ethanol," this volume). In the former case, chronic ethanol administration is associated with particular environmental cues, which the animal learns to associate with the effects of ethanol. When presented with the cues, the animal displays a compensatory response, which counteracts the effect of ethanol, thus producing tolerance. If the animal is given ethanol in an environment different from the one in which it has previously (chronically) received the drug, the compensatory response does not occur, and tolerance is reduced or absent. In contrast, environment-independent tolerance occurs regardless of the environment in which ethanol is chronically administered or in which the animal is challenged (see Tabakoff et al. 1982).

In our experiments, the tolerance maintained by AVP was environment independent. Since environment-dependent tolerance may be even more intimately linked with learning and/or memory, the effects of AVP on this type of tolerance should also be investigated if one wishes to evaluate similarities and differences between peptide effects on tolerance and on memory (see Melchior et al., "Influencing Environment-Dependent Tolerance to Ethanol," this volume, for preliminary results). The question of the relationships between tolerance and memory processes is also addressed by our structure-activity studies of neurohypophyseal peptide effects on ethanol tolerance (see below).

ROLE OF ENDOGENOUS AVP IN MODULATION OF ETHANOL TOLERANCE

A second issue raised by our findings is the possible role of endogenous AVP in the development or expression of ethanol tolerance. A related question is whether the site of action of the hormone for influencing tolerance is in the periphery or CNS. Several experimental approaches have been used to investigate the role of endogenous AVP in memory processes, and some of them may be valuable for studies of tolerance. De Wied and coworkers showed that intraventricular (ICV) administration of AVP antibodies had an effect opposite that of AVP on extinction of avoidance behavior (see van Ree et al. 1978). Studies with Brattleboro rats, a strain that lacks AVP, have, in some instances, shown that these rats have impaired memory, which can be improved by administration of AVP (see van Ree et al. 1978). These results are still somewhat controversial, however. More recently, an antagonist of the pressor and antidiuretic

effects of AVP was shown to have the opposite effects of AVP on extinction of active avoidance behavior in rats (Koob et al. 1981).

A recent study reported that Brattleboro rats did not develop tolerance to ethanol (Pittman et al. 1982), although blood-ethanol levels reached in these animals during ethanol exposure did not differ from those of controls. These results are in line with a role for endogenous AVP in tolerance development. The specific AVP antagonists may also provide a valuable tool for assessing the influence of endogenous AVP on ethanol tolerance; we are currently investigating the efficacy of such compounds to modulate the duration of ethanol tolerance.

SITE FOR AVP EFFECTS--CENTRAL OR PERIPHERAL

In the study of AVP antagonist effects on memory (i.e., avoidance behavior), a very high dose of the antagonist had to be given ICV to observe an effect (Koob et al. 1981). Another investigation showed that blockade of the peripheral (pressor) effects of AVP by a peptide antagonist also blocked AVP effects on memory (Le Moal et al. 1981). These findings suggested that the behavioral effects of AVP might result secondarily from the peripheral, endocrine actions of the hormone. This question has often been discussed since peripheral injection of very small amounts of AVP was reported to influence memory processes, and it is still not clear how easily this intact peptide can cross the blood-brain barrier.

There is evidence, however, that vasopressin modulates memory processes by acting at a central site. De Wied and his colleagues have found that AVP and various analogs are more potent when administered ICV rather than systemically (see van Ree et al. 1978). Further, analogs of AVP that have greatly reduced endocrinological activities (i.e., DGAVP and [des-9-glycinamide, 8-lysine]vasopressin [DGLVP]) are quite active in influencing memory-related functions (Walter et al. 1980). The study mentioned earlier, in which ICV, but not systemic, injection of anti-AVP antibody blocked AVP's effects on active avoidance behavior also suggests a central site of action for the hormone.

In addition, the ability of AVP to modify passive avoidance behavior can be blocked by lesions of specific noradrenergic systems in brain (Kovacs et al. 1979). The fact that blockade of peripheral effects of AVP by an antagonist also blocks AVP effects on avoidance behavior does not eliminate the possibility of a central site of action since the antagonist could simultaneously be blocking AVP's effects in the CNS. Similarly, although high ICV doses of vasopressin antagonist were necessary to demonstrate effects opposite AVP's on avoidance behavior, this does not necessarily indicate that the antagonist diffused to a peripheral site, as was discussed by Koob et al. (1981).

Structure-Activity Studies

The main evidence that AVP's effect on tolerance may be centrally mediated comes from our findings that both DGLVP, which we showed to be devoid of peripheral, endocrinological activities (see Walter et al. 1980), and DGAVP, which has also been reported to have reduced peripheral activity (Crabbe and Rigter 1980), were active in maintaining ethanol tolerance (Hoffman et al. 1980; Hoffman in press). We also carried out a more detailed study of the structural requirements for neurohypophyseal peptide maintenance of ethanol tolerance. The major objective of this investigation was to compare the requirements for modulation of ethanol tolerance to those for effects on active avoidance behavior and for attenuation of puromycin-induced amnesia (see Walter et al. 1980).

Our results indicated that the major structural requirement for peptide maintenance of tolerance is that the N-terminal "ring" portion of the peptide contain two amino acid residues with aromatic side chains. This structure is characteristic of vasopressin. However, the "ring" portion of the hormone per se was not active in maintaining tolerance, suggesting that the whole vasopressin molecule is necessary for maintenance of tolerance. These results were quite similar to those found both for peptide maintenance of active avoidance behavior and attenuation of puromycin-induced amnesia. However, peptides related structurally to the C-terminal portion of vasopressin or oxytocin were much more active in attenuating amnesia than in affecting active avoidance behavior and were relatively inactive in maintaining tolerance. In this respect, the ability of the peptides to maintain ethanol tolerance resembled their ability to inhibit extinction of active avoidance responses, an action that is thought to reflect peptide influences on memory consolidation. Thus, our results are in line with the idea that tolerance and memory-related processes may be influenced by similar CNS systems.

The structure-activity results also argue, although indirectly, for a central site of action of the peptides in modulating tolerance, even beyond the fact that DGAVP and DGLVP were active in this system. For example, the lack of effect of the peptide N-benzyloxycarbonyl-Pro-Leu-Gly-NH_2 (Z-PLG) and the relative inability of cyclo(Leu-Gly) to maintain tolerance (Hoffman in press) cannot be solely attributed to lack of uptake into brain since cyclo(Leu-Gly) was shown to accumulate in mouse brain after peripheral injection and Z-PLG was very potent in attenuating amnesia in mice (Walter et al. 1980). Further, the pattern of activities of the peptides in maintaining tolerance is somewhat different from the pattern observed for peripheral effects. For example, AVP and [8-lysin]vasopressin (LVP) were approximately equipotent in maintaining ethanol tolerance, while [8-arginine]vasotocin (AVT) was inactive. However, LVP and AVT are about equally active as pressor and antidiuretic agents in rats, and about half as potent as AVP (Berde and Boissonnas 1968). The

difference in activities when peripheral effects are compared and the similarities between peptide effects on tolerance and on maintenance of avoidance behavior--which, as described above, does appear to be mediated centrally--suggest that peptide maintenance of tolerance may also occur by putative CNS peptide-binding sites.

Neurochemical Studies

Another line of evidence for a central site of peptide action is provided by recent studies of the neurochemical mechanism by which vasopressin maintains tolerance to the hypnotic effect of ethanol. We had previously shown that intact noradrenergic systems are necessary for the development of ethanol tolerance in mice (Tabakoff and Ritzmann 1977). Partial destruction of these systems, before exposure of the mice to ethanol, blocked the development of tolerance. However, lesion of the noradrenergic systems after tolerance had developed did not affect the expression of tolerance.

Because of the involvement of noradrenergic systems in tolerance development, as well as in memory consolidation, it seemed reasonable that this neurotransmitter system might also contribute to AVP's modulation of ethanol tolerance. Mice were given the ethanol-containing liquid diet, such that ethanol tolerance was fully developed, and 24 hours after withdrawal from the diet, they were injected ICV with 6-hydroxydopamine (6-OHDA) or artificial cerebrospinal fluid (CSF). Groups of control mice were treated identically. Each group of mice was then subdivided into groups that received daily injections of saline or AVP (400 nmol/kg). When dissipation of tolerance was monitored, AVP maintained tolerance in the CSF-treated animals but not in the animals whose brain norepinephrine (NE) levels were depleted by 6-OHDA). Thus, intact CNS noradrenergic systems are necessary for AVP maintenance of functional ethanol tolerance (Hoffman et al. in press).

It is most interesting that destruction of the ascending dorsal brainstem noradrenergic neurons by 6-OHDA also prevented AVP maintenance of passive avoidance behavior (Kovacs et al. 1979). Vasopressin has been shown to influence catecholamine turnover in several brain nuclei (see van Ree et al. 1978); these results suggest that the hormone may well act directly in the CNS by modulating the activity of catecholaminergic neuron systems, thus influencing both tolerance and memory.

Other CNS neurotransmitter systems also appear to play a role in AVP's actions, however. Lesioning studies suggested that both noradrenergic and serotonergic systems mediate AVP maintenance of passive avoidance behavior in rats (Kovacs et al 1979). Partial destruction of serotonergic neurons also delays the acquisition of tolerance to ethanol in rats (Khanna et al. 1979). The development of ethanol tolerance in rats, in fact, appears to be

influenced by an interaction between noradrenergic and serotonergic systems (Le et al. 1981), with the serotonergic pathways being of primary importance.

In contrast, in the mouse, noradrenergic systems seem to be of primary importance for development of ethanol tolerance (Tabakoff and Ritzmann 1977). Very recently, Le et al. have demonstrated that in the rat, the vasopressin analog, DGAVP, can maintain ethanol tolerance (Le et al. 1982) and that depletion of serotonin blocks this ability of DGAVP (Le et al. 1982). Thus, serotonergic systems appear to mediate AVP's effects on tolerance in the rat. Because of the interaction between the two neurotransmitter systems with respect to ethanol tolerance in both the mouse and the rat (Le et al. 1981; Melchior and Tabakoff 1981), and with respect to AVP effects on memory in the rat, the role of each of these systems in mediating vasopressin effects on tolerance in the two species deserves further attention. We are currently investigating the importance of serotonergic systems in tolerance development, and vasopressin influences on tolerance, in the mouse.

CONCLUSION

The localization of vasopressin in brain areas outside the hypothalamus is now well documented (see Hoffman and Tabakoff 1981). It seems clear that there exist neurons containing vasopressin that are in a position to modulate the activity of various CNS neurotransmitter systems. Although the effect of ethanol on AVP release in the periphery is well known, little is known about ethanol's influence on AVP release in brain or into CSF. There is evidence that the normal regulation of central AVP release differs from that of peripheral release (Perlow et al. 1982). It will certainly be of interest to determine whether ethanol also influences central and peripheral release differentially and whether ethanol-induced alterations in CNS levels of AVP contribute to the development of tolerance to ethanol.

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Influencing Environment-Dependent Tolerance to Ethanol

Christine L. Melchior, Paula L. Hoffman, and Boris Tabakoff

INTRODUCTION

Tolerance to ethanol has been defined simply as a diminished response for a given dose of ethanol (Kalant et al. 1971). This definition encompasses such mechanisms as an altered central nervous system (CNS) response (functional tolerance) or changes in the absorption, distribution, or metabolism of ethanol (dispositional tolerance). Recently, it has become apparent that tolerance can be produced in a learning paradigm (environment-dependent tolerance), and this type of tolerance differs in certain features from tolerance produced in paradigms in which learning would not be likely to play a major role (environment-independent tolerance). Since various peptides have been shown to influence learning and memory (van Ree et al. 1978; van Wimersma Greidanus et al. 1975), tolerance produced in a learning paradigm should be susceptible to manipulation by these peptides.

ENVIRONMENT-DEPENDENT TOLERANCE

Environment-dependent tolerance can be produced in a classical conditioning procedure. In classical, or Pavlovian, conditioning, two stimuli are presented, such that the appearance of one reliably predicts the occurrence of the other. The first stimulus is called the conditioned stimulus; the second is the unconditioned stimulus. The unconditioned stimulus causes an action that is called the unconditioned response. After repeated pairings, the conditioned stimulus alone produces an action like the unconditioned response, which is then called the conditioned response. In the process of drug administration, the ritual surrounding the administration of the drug, as well as the environment in

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which it occurs, become conditioned stimuli, signaling the presence of the drug, the unconditioned stimulus. The pharmacological action of the drug is the unconditioned response. Although this definition suggests that the conditioned response will be similar to the effect of the drug, the opposite can also occur. That is, the conditioned response can be the opposite of the drug's action. In this paradigm, many drugs have been shown to cause such compensatory conditioned responses (Siegel 1977). If the compensatory conditioned response occurs when the drug is given, the result is a diminished response to the drug, which is tolerance.

Several investigators have demonstrated that tolerance to the hypothermic effect of ethanol can be produced in a conditioning paradigm (Le et al. 1979; Mansfield and Cunningham 1980; Crowell et al. 1981; Melchior and Tabakoff 1981c). As predicted by theories of conditioning (Kimble 1961), ethanol tolerance produced in this fashion can be extinguished effectively by an active extinction procedure, consisting of chronic saline injections in place of ethanol (Mansfield and Cunningham 1980).

NEUROCHEMICAL MECHANISMS THAT INFLUENCE ENVIRONMENT-DEPENDENT TOLERANCE

In our laboratories, we produced environment-dependent tolerance in C57B1 mice by injecting them twice a day for 4 days with 3.5 g/kg of ethanol in their home (cued) environment. Control mice received an equal volume of saline. On day 5, the animals were tested, either in the cued or a novel (uncued) environment. Ethanol-treated mice displayed tolerance to ethanol's hypnotic and hypothermic effects in the cued but not in the uncued environment. Saline-treated mice demonstrated a small hyperthermic response in the cued environment (i.e., a compensatory conditioned response) but showed no change from baseline in the uncued environment (Melchior and Tabakoff 1981c).

This paradigm was used to determine if manipulations of the catecholamine or serotonergic systems had the same effects on environment-dependent and environment-independent tolerance. When environment-independent tolerance is produced by a liquid diet technique (Ritzmann and Tabakoff 1976), prior administration of the catecholamine neurotoxin, 6-hydroxydopamine (6-OHDA), blocks the development of tolerance. This effect is due to the depletion of norepinephrine, not dopamine (Tabakoff and Ritzmann 1977). Preliminary data in our laboratory indicate that partial depletion of serotonin (5-HT) by the neurotoxin, 5,7-dihydroxytryptamine (5,7-DHT), also blocks the development of environment-independent tolerance. These observations on the effect of serotonin depletion on tolerance support the findings of the investigators at the Addiction Research Foundation (Le et al. 1981; Khanna et al. 1979).

We have also determined the effect of neurotransmitter depletion on the development of environment-dependent tolerance. When various doses of 6-OHDA were administered to mice 1 week before the start of the multiple-injection schedule, the rate of development of tolerance to ethanol's hypnotic effect was decreased in a dose-dependent manner (Melchior and Tabakoff 1981c). This decreased rate was related to the degree of norepinephrine, not dopamine, depletion. Thus, the effect of norepinephrine depletion, inhibition of tolerance development, is similar for both environment-dependent and environment-independent tolerance.

In the environment-dependent tolerance paradigm, disruption of the catecholamines might act to disrupt memory consolidation. The administration of 100 mg/kg of the catecholamine synthesis inhibitor, α-methyl-paratyrosine (AMPT), 2 hours after the second injection of ethanol each day of the multiple-injection schedule, also inhibited the development of tolerance to the hypnotic and hypothermic effects of ethanol (Melchior and Tabakoff 1981c). AMPT itself causes hypothermia, so that the daily injections of this compound may have provided additional learning trials for drug-induced hypothermia. However, since AMPT acts to inhibit, not to enhance, the rate of development of tolerance, its hypothermic effect was obviously not the critical point in determining its influence on tolerance development.

When 5,7-DHT was administered to mice 1 week before the start of the multiple-injection paradigm, initial sensitivity to the hypnotic effect of ethanol was increased, and the rate of development of tolerance on this measure was increased (Melchior and Tabakoff 1981c). Thus, the effect of 5-HT depletion on the development of tolerance depends on the type of tolerance being considered: the rate of development of environment-dependent tolerance is enhanced, and the development of environment-independent tolerance is inhibited.

A DISPOSITIONAL COMPONENT OF ENVIRONMENT-DEPENDENT TOLERANCE

In mice that show environment-dependent tolerance to ethanol, dispositional tolerance plays a significant role in the diminished response to ethanol. When ethanol-treated mice were tested for tolerance in the cued environment, analysis of blood-ethanol elimination data with the Widmark equation indicated that these animals had a larger volume of distribution than either ethanol-treated animals tested in the uncued environment or the corresponding saline-treated groups. As expected with a larger volume of distribution, brain-ethanol levels measured at 10, 30, or 60 minutes after the test injection were significantly lower in the animals that showed tolerance (Melchior and Tabakoff 1981a). When groups of animals received doses of ethanol from 1.5 to 3.5 g/kg during the training period, they demonstrated a training dose-dependent tolerance to the hypnotic and hypothermic effects of 3.5 g/kg ethanol delivered in the cued environment. Correspondingly, blood- and brain-ethanol levels obtained 30 minutes after

injection also showed a training dose dependence (Melchior and Tabakoff 1981b).

In assessing the role of tetrahydroisoquinolines (TIQs), both in the effects of ethanol and as neurotoxic pharmacological tools, the influence of some of these compounds on environment-dependent tolerance was tested. When the effect of intracerebrally administered TIQs was examined in mice, tetrahydropapaveroline and 3-carboxysalsolinol were found to block the development of environment-dependent tolerance. Blood and brain levels of ethanol in samples from these mice in the cued environment on the test day were significantly higher than the ethanol levels in blood and brain samples from mice that had received the control intracerebral (ICV) injection of an artificial cerebrospinal fluid (CSF) and did become tolerant (Melchior 1982). Although the mechanism involved in the cued alteration in volume of distribution is not known, these experiments indicate the involvement of a central component.

PEPTIDE EFFECTS ON ENVIRONMENT-DEPENDENT TOLERANCE

In considering the effects of neurohypophyseal peptides on environment-dependent tolerance, our first step was to examine the influence of these compounds on the acquisition of tolerance. The peptides chosen for examination were arginine vasopressin (AVP) and an analog that is devoid of peripheral endocrinological activities (Walter et al. 1975), [des-9-glycinamide, 8-lysine]vasopressin (DGLVP). These peptides have been shown to be effective in maintaining (or retarding the dissipation of) environment-independent tolerance in mice (Hoffman et al. 1978; Hoffman and Tabakoff 1981; Hoffman et al. 1980; Crabbe and Rigter 1980). With the use of the liquid-diet technique to produce environment-independent tolerance, tolerance was measured only after it had developed (i.e., initially at 24 hours after withdrawal of the ethanol-containing diet). While AVP and DGLVP did not affect the degree of tolerance measured at this time (Hoffman et al. 1978), this testing procedure allowed no assessment of effects on acquisition of tolerance. With the daily measurements obtained in the environment-dependent tolerance paradigm, effects of these peptides on acquisition can be evaluated.

The peptides were administered subcutaneously (SC) in a dose of 400 nmol/kg each day of the multiple-injection ethanol administration paradigm at 2 hours after the second daily dose of ethanol. Control mice received an equal volume of saline in place of peptide. This time of administration was selected on the premise that the peptides can act by influencing memory consolidation (van Ree et al. 1978).

Tables 1 and 2 show the "sleep time" and temperature data obtained after each morning injection of ethanol in the cued environment. The rate of acquisition of tolerance was decreased by

TABLE 1.--Effect of peptides on the acquisition of environment-dependent tolerance to the hypnotic effect of ethanol

Day	Sleep time (minutes)		
	Saline	AVP	DGLVP
1	31.33±0.26*	32.33±0.42	30.80±0.12
2	28.42±0.45	29.53±0.95	30.47±0.16†
3	24.67±0.27	26.20±0.39†	30.25±0.59†
4	21.65±0.24	24.38±0.25†	29.43±0.09†
5	20.36±0.21	22.68±0.35†	26.73±0.16†

*Mean ±SEM, N = 20.

†$p < .05$, t-test, compared with saline.

TABLE 2.--Effect of peptides on the acquisition of environment-dependent tolerance to the hypothermic effect of ethanol

Day	ΔT (°C)		
	Saline	AVP	DGLVP
1	3.66±0.07*	3.62±0.06	3.73±0.08
2	3.43±0.06	3.57±0.05†	3.74±0.12†
3	2.83±0.08	2.99±0.13†	3.22±0.07†
4	2.97±0.06	2.90±0.08	3.22±0.06†
5	2.60±0.07	2.94±0.08†	3.27±0.06†

*Mean ±SEM, N = 20.

†$p < .05$, t-test, compared with saline.

both peptides, with DGLVP being more effective than AVP. Since the peptides were administered in the cued environment, and AVP, like ethanol, causes an acute hypothermic response (Hoffman et al. 1980), these injections could be perceived as an additional training trial. If this were the case, one might expect AVP to enhance the rate of acquisition of ethanol tolerance, which it did not do. However, this may explain why AVP was less effective than DGLVP in decreasing the rate of tolerance acquisition.

The effect of AVP on extinction of environment-dependent tolerance was also examined. Mice were injected with 3.5 g/kg ethanol twice a day for 5 days in the cued environment. To extinguish tolerance, the mice were then given saline in place of ethanol twice a day in the cued environment. During the extinction period, half of the animals were also given 400 nmol/kg AVP, and half were given saline SC 2 hours after the second daily saline injection. At 1 week after the last ethanol injection, the hypnotic response to ethanol in the cued environment was again tested. Mice treated with AVP during the extinction period were significantly more tolerant ("sleep time" = 23.3±0.4 minutes, mean ±SEM; $\underline{N}$ = 15) than the mice treated with saline (sleep time = 29.0±0.4 minutes; $\underline{N}$ = 15). Thus, as with environment-independent tolerance to ethanol, AVP appears to inhibit the extinction of environment-dependent tolerance.

CONCLUSIONS

Environment-dependent tolerance can be influenced by various neurochemical manipulations. The mechanism for the effects of AVP and DGLVP in inhibiting acquisition of this type of tolerance are not readily apparent. Since AVP enhances catecholamine turnover in certain brain areas (Tanaka et al. 1977), one might expect it to have the opposite effect on tolerance from 6-OHDA, which inhibits the development of tolerance. However, it is clear that other neuronal systems also influence tolerance development, and the interaction of these systems may be important for determining the final effect of the peptides. On the other hand, the effects of AVP and DGLVP in inhibiting extinction of environment-dependent tolerance seem similar to their influence on extinction of other types of learning (van Ree et al. 1978; van Wimersma Greidanus et al. 1975) and on the dissipation of environment-independent tolerance to ethanol (Hoffman et al. 1978, 1980; Hoffman and Tabakoff 1981).

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Neuropeptides and Adaptive Responses to Ethanol: Vasopressin Modulation of Tolerance; Corticotropin Releasing Factor Antagonism of Ethanol-Induced Sedation

John R. Wenger

INTRODUCTION

Vasopressin ([8-arginine]vasopressin, AVP) and some of its analogs, have been reported to facilitate memory. In particular, AVP has been reported to both facilitate the acquisition of, and delay the extinction of, both passive (Ader and De Wied 1972) and active (De Wied 1971) avoidance responses in the rat. AVP also has similar effects on the development of tolerance to drugs. This is of interest because the development of drug tolerance has been reported to be, at least in part, mediated by learning processes (Siegel 1976; Wenger et al. 1980). Whereas AVP facilitates the acquisition of tolerance to morphine-induced analgesia (Krvoy et al. 1974), AVP-deficient rats (homozygous Brattleboro rats) became tolerant more slowly than non-AVP-deficient heterozygous controls (De Wied and Gispen 1976). Moreover, this deficiency in tolerance development was remedied by the administration of an AVP analog [des-9-glycinamide, 8-lysine]vasopressin (DGLVP) (De Wied and Gispen 1976). Similar effects on tolerance to ethanol have been reported. AVP facilitated the development of tolerance to the hypothermic effect of ethanol in the mouse (Rigter et al. 1980), and AVP-deficient rats became tolerant more slowly than nondeficient controls (Pittman et al. 1982). Further, daily administration of AVP, during and after withdrawal from ethanol, maintained the animal's tolerance to ethanol-induced hypothermia and sedation as long as AVP was administered (Hoffman et al. 1978).

In all of these studies, the behavioral measure of learning or tolerance was aversively motivated. In particular, in the ethanol-tolerance studies, the animals were either avoiding or escaping foot shock, hypothermia, or the supine posture. As of September 1982, there are no reports of which I am aware that are free

of motivational confounding, that AVP either facilitates the acquisition of or inhibits the extinction of, appetitively motivated behaviors. Thus, it appears that vasopressin facilitates the learning and retention of only aversively motivated behaviors. For this reason, we performed our first experiment to determine whether vasopressin would facilitate the acquisition of tolerance to ethanol as assayed by an appetitively motivated behavior.

VASOPRESSIN

THE EFFECT OF CONCURRENT AVP ON TOLERANCE DEVELOPMENT

In this experiment, food-restricted rats were trained to press an operant lever for food on a random-interval 30-second schedule of reinforcement. Then they were assigned randomly to one of six experimental treatment groups and given these treatments in conjunction with 10 additional days of training. Treatments consisted of administration of various combinations of either an injection of AVP or vehicle, immediately followed by an injection of either ethanol or saline. At 15 minutes later the animals were given a 15-minute opportunity to press a lever for food. Ethanol reduced the response rate as expected, but, surprisingly, so did AVP alone; moreover, these effects were additive. Tolerance developed over 10 days to all of these effects. On day 11, all of the animals were tested for tolerance to ethanol only. Only those animals that had received previously just ethanol were tolerant. The animals that had received AVP additionally were not tolerant. These differences were not due to differences in blood-ethanol concentrations.

These data are consistent with the hypothesis that AVP differentially affects the development of tolerance to ethanol, depending on whether the behavioral measure of tolerance is aversively or appetitively motivated (motivational specificity hypothesis). Thus, AVP may inhibit the development of tolerance to ethanol on appetitively motivated tasks, even though it facilitates the development of tolerance to ethanol on aversively motivated tasks, such as avoidance of foot shock and hypothermia.

Alternately, and/or additionally, the blockade of tolerance to ethanol by AVP observed in the present experiment can be interpreted in terms of learning theory. According to this interpretation, tolerance to ethanol may have developed in the animals that received both AVP and ethanol, but the tolerance became conditioned to only the stronger discriminative stimulus properties of the AVP but not to the weaker discriminative stimulus properties of ethanol; hence, it was not elicited or expressed during the tolerance test since the AVP cue was not present (overshadowing hypothesis).

BEHAVIORAL PHARMACOLOGY OF AVP

The observation that AVP reduced lever pressing motivated a series of experiments to investigate this effect further. AVP reduced the rate of lever pressing in a dose-dependent manner (data not shown). The dose that reduced the response rate to 50 percent was 4.53 nmol/kg (1 nmol is equivalent to 1.085 μg). The 6-μg/kg dose used previously was approximately in the middle of the dose-effect curve. A pressor antagonist of AVP dose-dependently antagonized the depressant effect of AVP on lever-pressing in a dose-dependent manner, but by itself it had no effect on the lever-pressing behavior. Intracerebroventricular (ICV) administration of the antagonist failed to block the effect of AVP administered subcutaneously (SC), suggesting that the AVP effect on lever pressing was mediated peripherally.

VASOPRESSIN, TOLERANCE, AND APPETITIVE MOTIVATION

Our next experiment on ethanol tolerance was designed to eliminate the possibility of overshadowing by administration of AVP after the daily practice sessions in the operant chambers. A total of 38 rats were handled and trained as before and then assigned randomly to one of four treatment groups. Two groups of 10 rats received an injection of ethanol (1.0 g/kg) 15 minutes before their operant session. The other two groups of nine rats each received the saline vehicle. One of each of the two ethanol groups received an injection of AVP (5 nmol/kg, SC) immediately after their daily operant session; the other groups received saline. The two groups that received saline instead of ethanol before their sessions were similarly divided, one receiving AVP, the other, saline, after their operant sessions. The tolerance-induction period was 5 days. Tolerance was tested on day 6. The results indicated that AVP had no effect on either tolerance or initial sensitivity to ethanol. These results are shown in figure 1. Subsequently, these animals were put into tolerance extinction where they received no presession injections of either ethanol or saline, but they did receive their usual postsession injections of either AVP or saline. The extinction period was interrupted to test for tolerance on days 3, 6, and 9. There was no reliable effect of AVP on tolerance on any of these days. The rats were then left untreated for 13 days, baseline measures were taken on day 22, and the rats were tested for tolerance on day 23. Again, there were no reliable effects of AVP on tolerance to ethanol.

We replicated this experiment with another group of rats using higher doses of ethanol to induce and test for tolerance. The tolerance-induction dose was 1.2 g/kg; the tolerance-testing dose was 1.6 g/kg. Again there was no reliable effect of prior AVP on tolerance. The animals were left untreated for the next 21 days except for days 6 and 20, when baseline measures of lever pressing were taken, and for days 7 and 21, when the animals

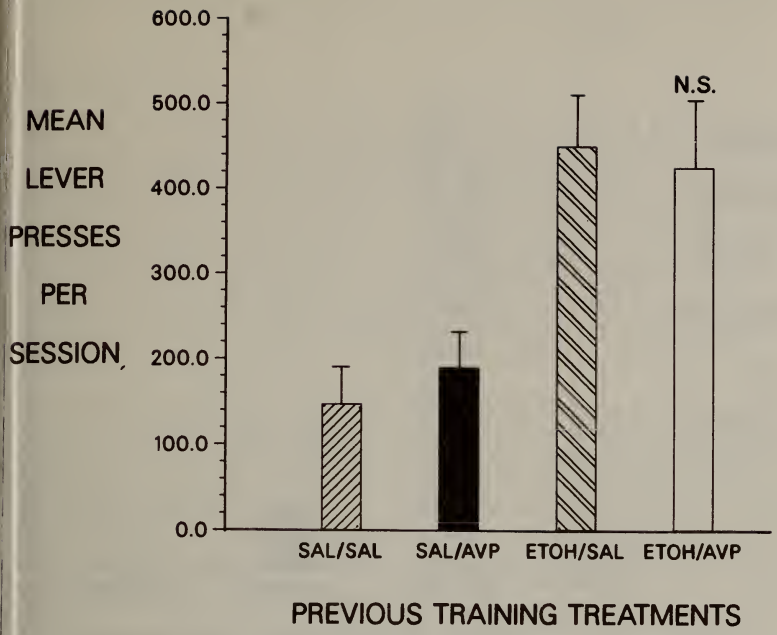

FIGURE 1.--Lack of effect of AVP on the development of tolerance to the disruptive effect of ethanol on an appetitively motivated behavior (values are mean ±SEM)

were tested for tolerance. These data suggest that AVP affects neither the development of tolerance nor the rate of loss of tolerance. Thus, these data do not support the general hypothesis that AVP facilitates all forms of learning. However, they are consistent with the motivational specificity hypothesis.

AVP, TOLERANCE, AND AVERSIVE MOTIVATION

To test the motivational specificity hypothesis directly, we studied the effect of AVP on the development of tolerance to the disruptive effect of ethanol on the performance of a two-way active avoidance task. Rats were first trained in a shuttlebox to avoid imminent foot shock that was signaled by a tone onset 50 seconds before the shock. If the rats failed to avoid the foot shock, they were shocked until they moved into the unshocked compartment or until 50 seconds had elapsed. Thus, each trial lasted a maximum of 100 seconds. A group of 17 trained rats were assigned randomly to one of three treatment groups, designated ETOH/AVP, ETOH/SAL, and SAL/SAL. Presession and postsession treatments are designated before and after the slash, respectively. The

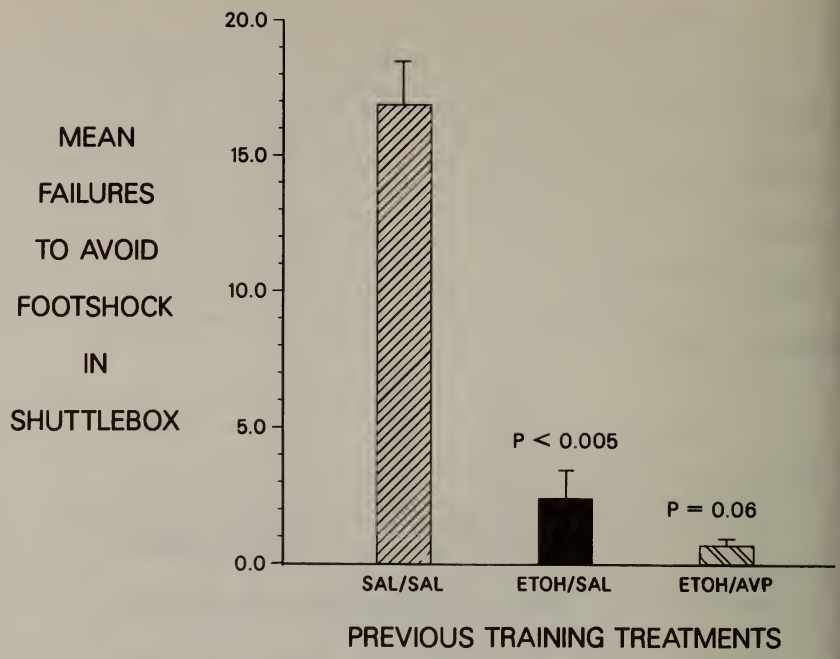

FIGURE 2.--AVP facilitates the development of tolerance to the disruptive effect of ethanol on an aversively motivated behavior (values are mean ±SEM)

tolerance-induction period was 5 days. Ethanol doses were 1.2 g/kg on day 1, 1.6 g/kg on days 2 to 5, and 1.8 g/kg on the tolerance-test day. Two measures of performance were analyzed: the number of failures to avoid foot shock and latencies to avoid or escape foot shock. On both measures the performance of the ETOH/AVP group was significantly better ($p < .06$) than the ETOH/SAL group, indicating that the AVP-treated animals were more tolerant than the non-AVP-treated animals. The avoidance data are shown in figure 2. Both ethanol-treated groups were significantly more tolerant than the saline-treated group ($p < .005$). This experiment is still in progress, and another five animals are scheduled for testing in each group. If the trend of the present results continues, the effect of AVP to facilitate tolerance to ethanol will become highly significant. The present data suggest that AVP facilitates the development of tolerance to the disruptive effect of ethanol on two-way active avoidance behavior. It is notable that this behavior is motivated by the aversive properties of foot shock. Thus, these data support the motivational specificity hypothesis. Most importantly, they are consistent with all of the previous reports (which used hypothermia and the righting reflex as their indexes of tolerance) that AVP

facilitates tolerance. It should be emphasized that both hypothermia and the supine posture are aversive to rats.

CONCLUSIONS

Doses of AVP commonly used to facilitate learned performance powerfully depress operant behavior. Further, AVP given concurrently with ethanol can prevent the development, as measured by the expression, of tolerance to ethanol as assayed by an appetitively motivated operant task. The stimulus properties of AVP may well mediate this effect. The depressant effects of AVP on operant behavior appear to be peripherally mediated. This raises the question as to whether the stimulus properties of AVP are mediated similarly in the periphery and whether they mediate the modulatory effects of AVP on memory processes.

AVP seems to facilitate neither the development, nor the resistance to extinction, of tolerance to ethanol-induced impairment of appetitively motivated behavior, strongly challenging the idea that vasopressin is intrinsically involved in the development and maintenance of tolerance. In contrast, AVP does seem to facilitate tolerance to ethanol-induced impairment of behaviors maintained by aversive motivation--i.e., AVP facilitation of tolerance is motivationally specific. This result is similar to the evidence that AVP facilitates aversively motivated tasks but not appetitively motivated learning tasks. Thus, the motivational specificity hypothesis of vasopressin modulation of memory processes appears to be supported by all of the currently known data. In particular, the vasopressin facilitation of the development of behavioral tolerance to ethanol, which may be regarded most parsimoniously as simply the manifestation of learning processes (Wenger et al. 1980), also appears to be motivationally specific.

What is the meaning of this motivational specificity? Five of the numerous hypotheses are discussed below. They are not mutually exclusive, and combinations of them are possible.

(1) Multiple substrates of learning hypothesis. There may exist multiple forms of learning, at least one aversively motivated, another appetitively motivated, and vasopressin may be a neurotransmitter in those brain areas that mediate the acquisition and retention of avoidance and escape behaviors but not appetitive behaviors.

(2) Generalized arousal hypothesis. Vasopressin may have aversive stimulus properties (see Koob's "Interaction of Vasopressin and Corticotropin Releasing Factor With Stress," this volume), and these may generally increase arousal, facilitating the acquisition and subsequent retention of avoidance and escape behaviors but not appetitive behaviors.

(3) Amplification of aversiveness hypothesis. Vasopressin may amplify the intrinsic aversiveness of external stimuli and

thereby increase the motivation to learn avoidance and escape behaviors but not appetitive behaviors.

(4) Addition of aversiveness hypothesis. The aversive stimulus properties of vasopressin may sum algebraically with those of the external stimuli, motivating avoidance and escape behaviors and thereby effectively amplifying the motivation to learn.

(5) Stress-mediation hypothesis. Vasopressin is known to release from the pituitary both ACTH and β-endorphin in equimolar amounts and was the standard of comparison for the even stronger recently characterized 41-amino acid, corticotropin releasing factor (CRF) (Vale et al. 1981). It is possible that the CRF-like (and perhaps CRF-releasing) properties of AVP may explain the motivationally specific effects of AVP on the development of tolerance to ethanol. Thus, we decided to assess the role of CRF in the modulation of the development of tolerance to ethanol.

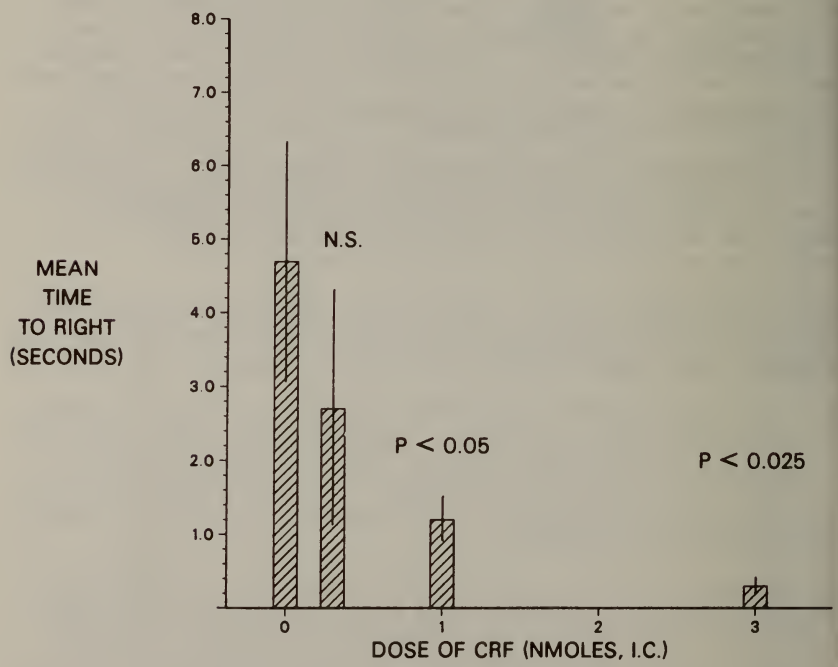

FIGURE 3.--CRF dose dependently antagonizes ethanol-induced sedation (values are mean ±SEM)

CRF AND INITIAL SENSITIVITY TO ETHANOL

CRF ANTAGONIZES ETHANOL-INDUCED SEDATION

In our preliminary experiments on the acute effects of CRF on the initial sensitivity to ethanol, rats were anesthetized with ether and then injected intracisternally with CRF (0.3, 1.0, or 3.0 nmol) or with the saline vehicle and subsequently with ethanol (3 g/kg) 30 minutes later. Ethanol-induced sedation was assessed at 1-hour intervals by measurements of the latency of the righting reflex. Rectal temperature was then recorded. These measures were recorded by an experimenter who was unaware of the previous intracisternal injections.

CRF dose-dependently antagonized the ethanol-induced sedation. Figure 3 shows the dose-effect relationship obtained 2 hours after the ethanol injection. This antagonism was time dependent. CRF (3 nmol) given immediately before the ethanol did not antagonize the ethanol-induced sedation, but CRF given 1 hour before

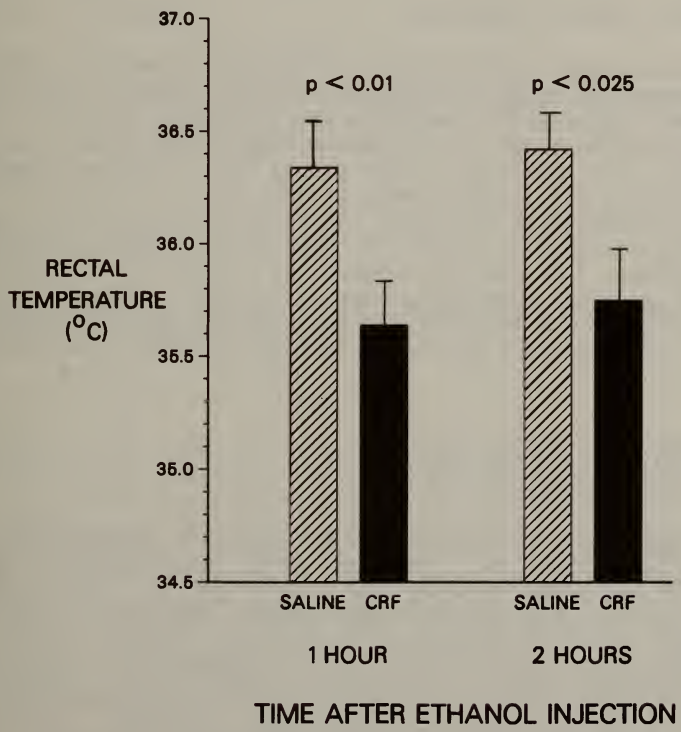

FIGURE 4.--CRF potentiates ethanol-induced hypothermia (values are mean ±SEM)

the ethanol did. Interestingly, CRF potentiated the ethanol-induced hypothermia (figure 4). Moreover, CRF dose-dependently induced hypothermia in the absence of ethanol (data not shown).

MECHANISM OF ACTION OF CRF ANTAGONISM OF ETHANOL-INDUCED SEDATION

Direct Neuroexcitation Hypothesis

The mechanism of the CRF-antagonism of ethanol-induced sedation is unknown. CRF directly excites at least some central nervous system (CNS) neurons. CRF excites hippocampal neurons in vitro within 3 minutes of CRF superfusion (Aldenhoff et al. 1982). ICV injections of CRF (3 μg) significantly increase the spontaneous firing rate of locus ceruleus neurons within 1 minute of the injection (R. Valentino and S. Foote personal communication, 1982). Thus, CRF may directly excite some neurons whose excitation counteracts ethanol-induced behavioral depression. Consistent with this is the observation that CRF (ICV) results in electroencephalographic activation (Ehlers et al. 1982).

Releasing-Factor Hypothesis

Alternately and/or additionally, CRF may act indirectly through the release of ACTH, β-endorphin from the anterior lobe of the pituitary, and α-MSH (melanophore stimulating hormone) from the intermediate lobe of the pituitary (Proulx-Ferland et al. 1982). However, our preliminary experiments with hypophysectomized rats indicate that hypophysectomy reduces, but does not eliminate the CRF antagonism of ethanol-induced sedation, thereby suggesting that the CRF is acting, at least partly, within the brain. Additionally, CRF may act centrally to release ACTH, β-endorphin α-MSH, or other substances from storage sites within the brain. The slow onset of action of CRF observed in the present experiments is presumably due to the diffusion of CRF to the sites of action.

Hypothermic Defense Against (Ethanol-Induced) Membrane Disorder Hypothesis

The potentiation by CRF of ethanol-induced hypothermia initially seems counterintuitive. However, in in vitro studies, ethanol-induced membrane disorder was greater at high than at low temperatures (Goldstein and Chin 1980). Moreover, membrane disorder could be induced in the absence of ethanol if the temperature were raised (Harris and Schroeder 1980). Therefore, hypothermia apparently functions to decrease the disordering effect of ethanol on cellular membranes. Therefore, hypothermia may be a defense against ethanol intoxication rather than merely an aspect of it. There are behavioral data to support this

conjecture (for references see Malcolm and Alkana 1981). Moreover, a primary purpose of body-temperature regulation may be the optimization of the biochemistry of cellular membranes. Specifically, membrane order may be regulated by body-temperature regulation. Thus, while ethanol-induced hypothermia may result passively from a disruption of temperature regulation, body cooling may also be an active defensive response against such membrane-disordering treatments as heat stress and depressant drugs, such as ethanol. CRF secretion may partially mediate ethanol-induced hypothermia. This hypothesis could be tested by antagonists, passive immunization, or desensitization procedures.

THEORETICAL IMPLICATIONS OF THE CRF EFFECTS

Homeostatic Regulation of Neuroexcitation Hypothesis

CRF may be a homeostatic regulatory response to counteract sedative-induced depression of the CNS.

Mediation of Initial Sensitivity Hypothesis

The setpoint of the regulatory mechanism controlling basal CRF release may determine, in part, the initial sensitivity to depressants. As a corollary, circadian variation of basal CRF release may contribute to the circadian variation of initial sensitivity to depressants.

Mediation of Acute Tolerance Hypothesis

Depressant-drug stimulation of CRF release may mediate the phenomenon of within-session tolerance.

Mediation of Tolerance Hypothesis

CRF secretion may mediate, at least in part, tolerance to ethanol and other CNS depressants. Although this mechanism might mediate both learned and (apparently) nonlearned tolerance, it is most interesting in the context of learned tolerance. Specifically, CRF secretion might, through the process of classical conditioning, become coupled to those stimuli (both exteroceptive and interoceptive) that reliably predict the sedative effects of ethanol and in the future be elicited by those (conditioned) stimuli. Thus, CRF secretion could become a classically conditioned compensatory response that opposes the sedative effects of depressants and thereby mediates learned tolerance to these effects (mediation of tolerance by classically conditioned neurohormonal secretion hypothesis). Similarly, secretion of CRF (or other neurohormones or

neurotransmitters) classically conditioned to the interoceptive stimuli associated with the effects of depressant drugs could mediate the apparently nonlearned tolerance that Tabakoff has heuristically termed "environment-independent tolerance" (see Tabakoff et al., "Evolving Concepts in Ethanol Tolerance and Dependence," this volume). However, classically conditioned secretion of CRF could not explain classically conditioned tolerance to ethanol-induced hypothermia since CRF itself results in hypothermia. Tolerance to the hypothermic effect of ethanol may develop secondarily through some other mechanism, and then only after tolerance has first developed to the membrane-disordering and sedative properties of the drug. The classically conditioned neurohormonal-secretion model of tolerance is quite general. Clearly, the release of other substances with anti-CNS-depressant effects (e.g., TRH [thyrotropin releasing hormone], epinephrine) might also be brought under stimulus control by means of classical conditioning. It appears that substances other than CRF mediate tolerance to depressant-induced hypothermia.

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Neurohypophyseal Peptides, Learning, and Drug Tolerance

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and Anh Dzung Lê

INTRODUCTION

Acquired functional tolerance to ethanol is defined as an increase in resistance to the intoxicating effects of ethanol on the central nervous system (CNS) after repeated exposure to this drug (Kalant et al. 1971). Although ethanol tolerance has received considerable attention over the past several decades, its mechanism is not fully known. Descriptive studies of ethanol tolerance in the past several years, however, have shown many similarities between tolerance and learning. Both have definable rates of acquisition and loss (Kalant et al. 1971). Both develop rapidly on successive cycles of exposure (Kalant et al. 1978), and both are markedly modified by environmental and behavioral factors (Kayan et al. 1969; LeBlanc et al. 1976; Siegel 1976). Recent work has even suggested that learning plays a key role in some manifestations of tolerance (Siegel 1978; Lê et al. 1979; Wenger et al. 1981). This notion of parallelism between tolerance and learning has served as a basis for many investigations into the possible involvement of neuronal systems in drug tolerance. The outcome of this approach has been quite successful. For example, inhibition of protein synthesis, lesions of the frontal cortex, or manipulations of neurotransmitters, such as norepinephrine (NE) or serotonin (5-HT), that have been implicated in learning processes, have been shown to influence tolerance development in a similar manner (for references see Kalant and Khanna 1980; Khanna et al. 1980, 1981; Kalant 1982). The basic rationale for experimental studies of the involvement of neurohypophyseal peptides such as vasopressin and oxytocin in opiate and ethanol tolerance is no exception. The reported effects of these peptides on learning processes have stimulated much interest in experimental investigations into their possible involvement in morphine tolerance. Since the work on ethanol tolerance has begun quite recently (Hoffman et al. 1978) and most of the work in this area is based on analogy to that of morphine and learning literature, an overview of peptide actions on learning as well as morphine tolerance is also covered.

INFLUENCES OF NEUROHYPOPHYSEAL PEPTIDES AND THEIR ANALOGS ON MEMORY PROCESSES

GENERAL EFFECTS

In the past decade much research has been devoted to examining the involvement of endogenous neurohypophyseal peptides in learning and memory processes. The literature on this subject reflects a multidisciplinary approach in addressing the problem. Much of this literature has been the subject of recent book chapters and comprehensive reviews (De Wied and Gispen 1977; van Ree et al. 1978; van Ree and De Wied 1980; Kovacs et al. 1979a; Bohus et al. 1982). In this section, this work is briefly summarized; for more detail readers are referred to the reviews mentioned above.

De Wied (1965) demonstrated that removal of the posterior pituitary gland results in an impairment of avoidance learning, which can be restored by treatment with pitressin or [8-lysine]vasopressin (LVP). In subsequent work, De Wied and coworkers reported that certain fractions extracted from porcine pituitary can restore the ability to acquire conditioned avoidance response by hypophysectomized rats (Lande et al. 1971; De Wied et al. 1972). One of these fractions is [des-9-glycinamide, 8-lysine]vasopressin (DGLVP), an active component of vasopressin with minimal endocrine activity (De Wied et al. 1972). Since then, the administration of various vasopressin analogs, most commonly, DGLVP and DGAVP (des-glycinamide arginine vasopressin), either by peripheral or central routes, has been shown to influence the retention and retrieval of various learning tasks (De Wied 1976; De Wied and Gispen 1977; Bohus 1976; Koob and Bloom 1982). Additional support for the involvement of vasopressin in memory is derived from studies with experimentally induced amnesia in mice and rats. Administration of puromycin, a protein synthesis inhibitor, or inhalation of CO_2 abolished the acquired learning task in animals. Vasopressin or DGLVP administered before learning or before the test trial can protect or reduce the amnesia induced by such procedures (Lande et al. 1972; Walter et al. 1975; Rigter et al. 1974).

To demonstrate the involvement of endogenous vasopressin in the regulation of memory processes, a different experimental model was used. Bohus et al. (1975) reported that diabetes insipidus rats (Brattleboro strain), which lack the capacity to synthesize vasopressin, were inferior in acquiring or maintaining active and passive avoidance behavior compared with normal control rats. It was also shown that administration of DGLVP can restore or normalize learning deficits in these animals (De Wied et al. 1975). In addition, central blockade of vasopressin activity by intraventricular (ICV) injection of vasopressin antiserum after the learning trial also resulted in a deficit in the retention of learning behavior (van Wimersma Greidanus et al. 1975).

The involvement of oxytocin in memory processes has also been examined, although less extensively compared with vasopressin. In general, small doses of oxytocin or its analogs mimic the effects of vasopressin on the maintenance of conditioned avoidance behavior (De Wied and Gispen 1977). However, higher doses of oxytocin or administration of oxytocin antiserum reportedly produce effects opposite that of vasopressin or its antisera, respectively, in avoidance learning behavior (De Wied and Gispen 1977; van Ree et al. 1978). It has been suggested that centrally released oxytocin may be a naturally occurring amnesiac neuropeptide (Bohus et al. 1978).

SITES AND POSSIBLE MECHANISMS OF ACTION

Specific evidence has been presented for the sites of action of vasopressin in the brain. Rats with lesions in the parafascicular area were shown to be less sensitive to the effect of vasopressin on the extinction of a conditioned avoidance behavior (van Wimersma Greidanus et al. 1974). Depending on their size, lesions in the anterodorsal hippocampus partly inhibited or completely prevented the effect of vasopressin on the extinction of avoidance behavior, but they had no effect of their own on extinction of behavior, as shown by van Wimersma Greidanus and De Wied (1976). These authors suggested that this region is one possible site that mediates the behavioral action of vasopressin and that an intact limbic system appears necessary for the effect of vasopressin. However, recent work by the same investigators suggests that the action of [8-arginine]vasopressin (AVP) may be much more localized than previously thought. Fornix transection, which destroys all the connections between the limbic system and the hypothalamus as well as all hippocampal efferents and direct septal input to the hippocampus, abolished all the effect of ACTH but not that of vasopressin on the extinction of avoidance response (van Wimersma Greidanus et al. 1979). Direct administration of AVP into hippocampal dentate gyrus, dorsal raphe nucleus, or dorsal septal area facilitated retention of passive avoidance behavior. Similarly, the administration of AVP into the central nuclei of amygdala or locus coeruleus produced no demonstrable effect on such measure (Kovacs et al. 1979a; Bohus et al. 1982). Direct administration of oxytocin in these areas produced effects opposite that of vasopressin in the extinction process, except in the dorsal septal area, where both peptides facilitated the retention of such learning (Bohus et al. 1982). Additional evidence for the involvement of the hippocampus in the action of vasopressin is derived from studies with vasopressin antiserum. Direct administration of AVP antiserum into the dentate gyrus of the dorsal hippocampus has recently been shown to attenuate the retention of avoidance learning (Kovacs et al. 1982).

INTERACTION WITH MONOAMINERGIC SYSTEMS

Both brain norepinephrine (NE) and serotonin (5-HT) have been implicated in the behavioral action of vasopressin. Evidence for the involvement of NE in vasopressin action is derived from several reports. First, the administration of vasopressin has been shown to interfere with the disappearance of NE in several limbic areas induced by α-methylparatyrosine (Kovas et al. 1979a). Second, the catecholamine turnover in these regions was much lower in vasopressin-deficient rats than in controls (Versteeg et al. 1978). Finally, destruction of the dorsal noradrenergic bundle by injection of 6-hydroxydopamine (6-OHDA) prevented the consolidation effect and, to a lesser extent, the retrieval effect of vasopressin (Kovacs et al. 1979a; Bohus et al. 1982). Similar lesions of the dopaminergic system or serotonergic pathway originating from the dorsal raphe nucleus did not influence the effect of AVP on the consolidation process (Bohus et al. 1982). Surprisingly, direct injection of vasopressin or vasopressin antiserum into the dorsal raphe nucleus resulted in the facilitation or attenuation of the consolidation process (Bohus et al. 1982). However, the effect of vasopressin, or its antiserum, in the dorsal raphe, can be prevented by destruction of NE terminals in this area, as shown by Bohus et al. (1982) and Kovacs et al. (1979b). These workers suggested that a serotonergic pathway originating from the dorsal raphe might play a secondary role in the mechanism of action of AVP on memory consolidation.

There are, however, several apparent contradictions as well as difficulties in interpreting these data. First, it is known that direct injection of AVP or its antiserum into the dorsal raphe nucleus can mimic or antagonize the effect of peripherally administered AVP, respectively, while destruction of this nucleus does not produce any behavioral effect or antagonize the behavioral action of peripherally administered AVP. Second, as mentioned, the dorsal hippocampus seems to be an important site for AVP action, yet this area receives most, if not all, of its 5-HT input from the median rather than from the dorsal raphe nucleus (Jacobs et al. 1974; Geyer et al. 1976). Third, although lesions of the dorsal noradrenergic bundle inhibit the facilitory effect of AVP on consolidation processes, lesioning of this pathway by itself does not affect such processes to any marked degree. Therefore, these two systems may act independently to modulate consolidation processes.

Besides the data mentioned above, evidence for the involvement of brain 5-HT in AVP-mediated behavioral effects was provided earlier. Rigter et al. (1975) reported that learning of a footshock avoidance task is associated with an increase in hippocampal 5-HT. This change in 5-HT content, however, disappeared when amnesia was induced by CO_2 inhalation. In subsequent work, Ramaekers et al. (1977) demonstrated that the anti-amnesiac action of DGLVP is associated with an increase in hippocampal 5-HT content. Auerbach and Lipton (1982) have also provided

some evidence for the possible interaction between AVP and 5-HT in the hippocampal area. Incubation of hippocampal slice preparation in the presence of AVP resulted in an increase in the release and the synthesis of 5-HT during depolarization of these nerve terminals. The studies described above suggest that vasopressin might influence memory processes by exerting its action on certain specific loci in the limbic area. In addition, brain NE and 5-HT might interact with vasopressin in modulating memory processes. The exact loci or manner in which these systems interact with one another to modulate such processes, however, needs to be elucidated.

NEUROHYPOPHYSEAL PEPTIDES AND MORPHINE TOLERANCE AND PHYSICAL DEPENDENCE

Krivoy et al. (1974) were the first to investigate the influence of a vasopressin analog (DGLVP) on the development of tolerance to morphine. They reported that administration of DGLVP (50 μg/mouse) facilitated the acquisition of tolerance to the analgesic effect of morphine. A close examination of these data revealed two interesting phenomena. First, in the animals that received high daily doses of morphine, DGLVP enhanced the rate of tolerance acquisition without affecting the final level of tolerance attained. Secondly, although there was no apparent tolerance in animals that were treated daily with test doses of morphine (8 mg/kg), tolerance to morphine analgesia was evident in the group that received test doses of morphine and DGVLP. Surprisingly, this enhancement of tolerance to the test dose of morphine reached a maximum after the second morphine injection. Further, the level of tolerance was quite comparable with that of the group that received high daily morphine treatment. Additional evidence for the involvement of vasopressin in morphine tolerance was subsequently provided by De Wied and coworkers. De Wied and Gispen (1976) demonstrated that diabetes insipidus rats, which lack the ability to synthesize vasopressin, acquired tolerance to morphine at a much slower rate than normal animals. In the same study, these workers also showed the administration of vasopressin or DGLVP to these animals normalized their rate of tolerance acquisition. Similarly, ICV administration of vasopressin antiserum has also been reported to inhibit the development of tolerance to morphine-induced analgesia (van Wimersma Greidanus et al. 1978).

Tolerance and physical dependence are usually regarded as two aspects of the same phenomenon (Kalant et al. 1971). Sensitivity to naloxone-induced loss in body weight and reduction in body temperature after morphine treatment has been demonstrated by Takemori (1975) as a good indicator for the extent of tolerance to, and dependence on, morphine. Using the Takemori test, van Ree and De Wied (1976, 1977) reported that DGAVP treatment facilitated the development of tolerance to, and physical dependence

on, morphine. They also reported that although oxytocin was less active in affecting behavior at a dosage comparable with vasopressin, it was much more potent in facilitating the development of tolerance and dependence to morphine. Further, of all the analogs tested in these studies, PLG (prolyl-leucyl-glycinamide) the C-terminal tripeptide of oxytocin was the most potent. This effect of PLG on the development of tolerance to, and physical dependence on, morphine has also been observed by other investigators (Contreras and Takemori 1980).

Failure to demonstrate any observable effect of these peptides on morphine tolerance (Schmidt et al. 1978) or on morphine tolerance and dependence has also been reported (Mucha and Kalant 1979). Schmidt et al. (1978) did not observe any effect of vasopressin or oxytocin treatment on the final level of analgesic tolerance to morphine induced by pellet implantation or by daily morphine injection. The failure to observe any effect of these peptides on morphine tolerance in this study may have been due to the fact that tolerance testing was done only once, on the final day of treatment. It is quite likely that this morphine regimen produced maximal tolerance at the time of testing. As mentioned above, DGLVP treatment appears to accelerate the rate of tolerance development rather than its final extent. It is difficult to reconcile the differences in the findings of van Ree et al. (1978) and of Mucha and Kalant (1979) on the effects of PLG on morphine tolerance and dependence. Except for sex differences, the test procedure as well as the doses of PLG or morphine used in these two studies are the same. In addition, in the study by Mucha and Kalant (1979), despite the use of various PLG and morphine doses, no detectable effect of PLG on dependence was observed. However, in recent work by Walter et al. (1979), PLG treatment resulted in the inhibition, rather than facilitation, of tolerance development.

It is clear from the data that when morphine tolerance was assessed by a reduction in the analgesic response to a challenge dose of morphine, a consistent picture of peptide effect was observed. The administration of exogenous vasopressin or its fragments factilitated the development of tolerance, but vasopressin-deficient animals acquired morphine tolerance at a much slower rate. The use of naloxone challenge in assessing the changes in morphine tolerance and dependence, after peptide treatment, however, is complicated in that no consistent picture has emerged. Future work is clearly needed before any definite conclusions can be drawn regarding the action of peptides on morphine tolerance and dependence. Moreover, other tests such as catalepsy should be used to determine whether the effect of peptides can be generalized to other drug effects on neuronal systems.

Hoffman et al. (1978) examined the effect of vasopressin and oxytocin on the decay of ethanol tolerance. In this study, mice were rendered tolerant to ethanol by exposure to a liquid diet containing ethanol for 7 days. The retention of ethanol tolerance, as measured by ethanol-induced hypothermia or sleeping time, was assessed at 3-day intervals after ethanol treatment ended. By day 6 after ethanol withdrawal, tolerance was no longer observed in saline-treated or oxytocin-treated animals. On the other hand, animals that received vasopressin treatment during tolerance induction as well as after ethanol treatment retained their tolerance. In subsequent work, Hoffman et al. (1979) also reported that even if vasopressin treatment was restricted only to the period of ethanol withdrawal, a similar effect of vasopressin on the retention of ethanol tolerance was observed. Such tolerance, however, disappeared once the peptide treatment was discontinued. Similar effects on the retention of ethanol tolerance have been reported following the administration of vasopressin analogs (DGLVP and DGAVP) in the mouse and rat (Rigter et al. 1980a,b; Hoffman et al. 1980; Lê et al. 1982). In the study by Rigter et al., administration of DGAVP enhanced the residual tolerance to ethanol. However, in contrast to the work by Hoffman et al. (1978), such tolerance was not completely maintained despite the continuation of DGAVP treatment. The apparent difference in the degree of DGAVP action in the retention of ethanol tolerance might be due to the difference in the rate of tolerance loss observed in these studies. The efficacy of DGAVP in retaining ethanol tolerance might be related to the rate of tolerance loss. When tolerance decayed quite rapidly, as in the case of tolerance to hypothermia, DGAVP administration only retained part of the initial tolerance. On the other hand, when tolerance disappeared slowly, as in the case of motor impairment, DGAVP retained all the tolerance that was acquired (Lê et al. 1982).

These studies also suggest that the effect of vasopressin on the retention of ethanol tolerance can be generalized to several drug effects. The retention of ethanol tolerance by DGAVP was observed in the mouse and rat as well as across different testing paradigms such as hypothermic, hypnotic, and motor-impairing effects of ethanol. This effect of DGAVP or AVP on ethanol tolerance is not mediated via its interference with the pharmacokinetics of ethanol. In all these studies, AVP or DGAVP administration did not alter blood-ethanol levels measured at various intervals (Hoffman et al. 1978, 1979, 1980; Rigter et al. 1980a,b; Lê et al. 1982). Other analogs of oxytocin, such as PLG and cyclo(leucyl-glycine) have also been shown to produce no detectable effects on ethanol tolerance. However, oxytocin administration has been reported to interfere with the initial response to the hypothermic effect of ethanol (Rigter et al. 1980a).

The above studies indicate that administration of vasopressin or its analogs can enhance the retention of the acquired ethanol

tolerance. The effect of these peptides on the rate of acquisition of tolerance to ethanol, however, has not been clearly demonstrated. Kalant (1978) reported that DGLVP treatment in a low dose (0.2 μg) enhanced the development of behaviorally augmented tolerance to ethanol as measured by the moving-belt test. However, with a higher dose of this peptide (2.0 μg), an inhibition of tolerance was observed. On the other hand, administration of vasopressin (6 μg/kg) has recently been reported (Wenger et al. 1982) to inhibit the development of tolerance to ethanol, as measured by its disruptive effect on operant behavior. It is difficult to draw any conclusion from these studies concerning the action of vasopressin on the acquisition of ethanol tolerance because various studies used different doses of AVP or its analogs and different tests to measure tolerance. A recent study by Pittman et al. (1982), however, suggested that endogenous vasopressin may be important for ethanol tolerance. These authors demonstrated that despite prolonged ethanol treatment, the Brattleboro rats that are deficient in vasopressin synthesis failed to develop tolerance to the hypothermic effect of ethanol. Similar treatments resulted in tolerance development in the normal rats.

Evidence has also been presented for the interaction between the mesolimbic brain 5-HT pathway, which originates from the median raphe, and vasopressin in the retention of ethanol tolerance (Lê et al. 1982). Although administration of DGAVP to sham-operated or dorsal raphe-lesioned rats retained ethanol tolerance, no such effect was observed in the median raphe- or joint median plus dorsal raphe-lesioned animals. Since destruction of the mesolimbic 5-HT pathway by itself also accelerated tolerance loss, the failure for DGAVP to retain or influence such effect might suggest that an intact mesolimbic 5-HT pathway is necessary for the action of DGAVP on the retention of ethanol tolerance. Recently, Hoffman et al. (1982) have suggested that the brain noradrenergic system is also involved in a vasopressin-mediated retention of ethanol tolerance. Previous work from our laboratory has demonstrated an interaction between NE and 5-HT system in modulating ethanol tolerance (Lê et al. 1981). Thus it is possible that vasopressin, 5-HT, and NE systems operate in a complex network to regulate ethanol tolerance.

CONCLUSIONS

The data reviewed clearly demonstrate that endogenous vasopressin or its analogs are quite important for adaptive processes and drug tolerance. Evidence to support this notion is derived from studies with rats that are genetically vasopressin deficient. These animals demonstrated an impairment in their acquisition of a newly learned task (De Wied and Gispen 1977; van Ree et al. 1978) and tolerance to morphine (De Wied and Gispen 1976) and to ethanol (Pittman et al. 1982). The impairment of learning and drug tolerance was also shown to be reversed by administration of vasopressin. In contrast to vasopressin, the role of exogenous oxytocin in these processes has not been fully investigated.

The available evidence, however, suggests that it might have a function opposite that of vasopressin (van Ree et al. 1978; van Ree and De Wied 1980).

Clearly, vasopressin facilitates learning processes as well as drug tolerance. However, the characteristics of such effects are not quite the same. In both learning and ethanol tolerance, vasopressin appears to exert its effect primarily on the retention of these processes but not on the acquisition phase. For morphine tolerance, however, quite a pronounced effect on acquisition was observed (Krivoy et al. 1974). Whether or not vasopressin administration would influence the retention of morphine tolerance still remains to be investigated. Similarly, although oxytocin and its analogs were shown to be involved in learning processes (van Ree et al. 1978), they produced no apparent effect on ethanol tolerance (Hoffman et al. 1978, 1980).

Work in both areas, however, suggests that both brain NE and 5-HT are involved in vasopressin-mediated effects. There are, however, certain important aspects regarding the possible sites and mechanism of vasopressin actions that can be derived from the literature. First, the hippocampus is an important site of action of vasopressin (van Wimersma Greidanus and De Wied 1976; Kovacs et al. 1982). Second, there is quite a close association between hippocampal 5-HT metabolism and the anti-amnesiac action of vasopressin (Ramaekers et al. 1977). Third, a direct biochemical interaction between vasopressin and 5-HT has been demonstrated (Auerbach and Lipton 1982), and hippocampal 5-HT plays an important role, possibly postsynaptically, in the retention of ethanol tolerance by vasopressin. Finally, recent work by Gage and Springer (1981) has demonstrated an interaction between 5-HT and NE in the hippocampal area in regulating behavioral processes. These factors strongly suggest that the interactions among vasopressin, 5-HT, and NE in the hippocampus may provide an attractive model for future work in neurochemical mechanisms of learning processes and drug tolerance. It would also be important to determine whether certain brain structures that have been implicated in vasopressin effects on learning are also involved in drug tolerance.

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Discussion of Section

Harold Kalant

Throughout the chapters in this section, there are some closely related questions, presented from different points of view. They are important and fundamental questions that can act as guides to future research in this field:

(1) Are there really two or more different forms of tolerance, some of which are environment dependent and others that are environment independent?

(2) How do the processes of learning, including classical and operant conditioning, relate to alcohol tolerance, especially to an environment-independent tolerance if this indeed exists?

(3) What role do endocrine factors play in each type of tolerance?

(4) What is the mechanism by which the various neuropeptides affect the acquisition or retention of tolerance, and is this mechanism a central or peripheral one?

(5) How do the environmental and neuroendocrine influences on tolerance relate to the membrane mechanisms and other cellular alterations described by Goldstein?

It is useful to recall the rhetorical question posed by Bloom: what use is tolerance? Researchers in the alcohol and drug field now probably accept the fact that tolerance is an adaptive or homeostatic response to functional disturbances produced by drugs. Therefore, alcohol tolerance and other types of drug tolerance belong in the general category of adaptive responses, which also includes learning and various physiological adaptations, such as those to alterations in environmental temperature, salinity, and pH. Moreover, very few investigators would now argue against the concept that learning requires a physiological substrate and that therefore psychological processes at some point must have a physiological explanation at the cellular level. The question remains, however, whether alcohol tolerance shares a common mechanism with these other types of functional adaptation or whether it is merely a formal analogy to them. How do they interact?

Tabakoff discusses different neurophysiological mechanisms that might underlie environment-dependent and environment-independent

forms of tolerance. I have great difficulty in conceiving of any tolerance that is truly independent of the environment. The capacity for developing tolerance to alcohol and other drugs seems to be inherent in all living organisms, even unicellular ones. In such organisms, it is extremely difficult to conceive of a difference between physiology and learning, since adaptations to environmental disturbances in single-celled organisms are explicable only in terms of cellular physiology. In multicellular organisms, the internal environment is equivalent to the external environment of unicellular organisms, and the true external environment in multicellular organisms is translated into changes in the internal environment that impinges directly on individual cells. This internal environment is always present and must still be altered by the effects of drugs, even if it were possible (which is really questionable) to exclude completely the influences of the external environment. It also seems improbable that responses to the internal environment are mediated by different neural and endocrine mechanisms than those invoked in response to the external environment. Therefore, in all probability the distinction between environment-dependent and environment-independent forms of tolerance in higher organisms is not an absolute one, but it reflects a greater complexity of modulating factors when external and internal environmental factors come into play. The two types of environmental factors can apparently be influenced differentially by different manipulations, but they ultimately act on the same final cellular mechanism or process to produce the tolerance.

Melchior mentions the effects of classical Pavlovian conditioning on the production of alcohol tolerance. The role of such conditioning in drug tolerance, which has been studied in great detail by Siegel (1976) and others, is obviously of considerable importance, but its mechanism remains to be established. One aspect of this model must be emphasized. In most instances of classical conditioning, the conditional stimulus that is paired with the unconditional stimulus comes, in time, to elicit the same effects as those of the unconditional stimulus. On this basis, we would expect that the conditional stimuli paired with drug administration should come to elicit the typical effects of the drug. Instead, we find that it is the adaptive response to the initial effects of the drug that is ultimately elicited as the conditional response. It is obviously implicit in the model, therefore, that a fundamental physiological adaptive response to the drug effects exists as an unconditioned reaction before the conditioning process comes into play.

It seems reasonable, therefore, to suggest that the effects of endocrine and learning factors are exerted on a common basic adaptive response and serve to modify its rate, extent, and duration but not to change its basic character. If this is true, it removes one source of difficulty in relating the modification of tolerance in the intact organism to alterations in cell membranes and other functional changes studied in isolated preparations.

It has become increasingly clear in the past few years that tolerance is not a single phenomenon that applies equally to all the effects of a given drug. For example, Wenger advances the interesting hypothesis that vasopressin-related peptides enhance the development or retention of tolerance to the effects of alcohol on aversive tasks but not to those of appetitive tasks. Hoffman describes changes in tolerance produced by the manipulation of catecholaminergic pathways in the brain, and Khanna outlines the effects of modification of serotonergic pathways. There are probably many other neurotransmitter modifications that can alter the rate and extent of development of tolerance to various individual functional disturbances produced by alcohol. Thus, many different neuronal pathways are probably involved in the development of tolerance, depending on which function is used as the test for tolerance development. It is equally possible that endocrine modulators, including neuropeptides, steroids, and autacoids, may also act differentially, depending upon the particular neuronal pathway and the particular test function under study.

Koob raises the important question of whether or not peptides and other endocrine factors act peripherally or centrally. Clearly the more complex the organism is, the larger the number of available means and pathways by which environmental and endocrine factors might influence central processes. As Koob suggests, elevation of blood pressure by the pressor effect of vasopressin could alter information carried by neuronal pathways from the periphery to the brain, and thus modify adaptive processes in the brain by secondary or tertiary or even more remote effects. Nevertheless, there does appear to be sufficiently strong evidence to argue that at least a part of the effect must be exerted centrally.

De Wied's group in Utrecht showed that vasopressin, and vasopressin derivatives, such as DGAVP (des-glycinamide arginine vasopressin) and DGLVP ([des-9-glycinamide, 8-lysine]vasopressin), produced the same retention of conditioned avoidance behavior during extinction trials, regardless of whether a dose in micrograms was administered peripherally or a dose in nanograms was administered intraventricularly (ICV) (De Wied 1976). In this section, Khanna discusses the requirement for an intact mesolimbic serotonin pathway for DGAVP to produce retention or prolongation of alcohol tolerance. Although this does not prove a central action of the peptides, it is consistent with the observations by Ramaekers et al. (1977) that intraventricular action of the peptide modified hippcampal serotonin levels. Earlier work (Dell 1972; Lacey 1972) argues against an indirect, peripheral mechanism of action of vasopressin by stress-induced arousal since elevation of the blood pressure peripherally tended to reduce, rather than to increase, the arousal level. Therefore, although peripheral influences cannot be excluded, there seems to be at least a major action by vasopressin-related and other peptides at central loci.

One puzzling discrepancy must be pointed out--that between the effects of thse peptides described in this section and some earlier observations. In our laboratory, as Khanna described, we found that the effects of DGAVP are much stronger on the retention of tolerance than on its acquisition, just as Hoffman et al. (1978) and Rigter et al. (1980) found. Yet in separate studies, we observed a clear effect of DGAVP on the acquisition of alcohol-drinking behavior in the rat. In separate experiments, both Finkelberg et al. (1978) and Mucha and Kalant (1979) found that DGAVP, given during the acquisition phase, resulted in a higher and better maintained intake of alcohol by the rat and acceptance of a higher alcohol concentration in the drinking water. The model used in those studies was an operant model in a sense. The acceptance or rejection of a given alcohol solution by the rat directly determined the change in concentration of alcohol presented on subsequent days. It is conceivable, therefore, that the effect of the peptide on the acquisition of alcohol-drinking behavior was an influence on learning.

In contrast, the reported studies of the effects of the peptide on ethanol tolerance relied principally on the use of alcohol-induced hypothermia as the test measure for tolerance (Hoffman et al. 1978; Le et al. 1982). Possibly the use of different test behaviors, with varying components of learning in the acquisition of tolerance, might help to resolve the apparent discrepancy.

For all of these reasons, there is a clear need for an extensive and detailed program to map the effects of endocrine factors on tolerance that is induced by a wide range of different alcohol doses, by different routes of administration, and on different types of test performance. Such mapping, by providing much more specific indications of the type and degree of peptide effect in different models, may well help to eliminate overly simplistic global concepts of endocrine effects on tolerance and to replace them by more specific suggestions as to possible sites and mechanisms of action.

There remains the fundamental question of how environmental and endocrine influences are related to basic cellular mechanisms. The finding of significant changes in structure and function of neuronal cell membranes in animals that are chronically exposed to alcohol does not necessarily prove that such changes represent the ultimate cellular mechanism of tolerance. These changes are seen after chronic alcohol exposure and thus might be expressions of tolerance, or even consequences of tolerance, rather than mechanisms that give rise to tolerance. To establish a causal or mechanistic link, it is necessary to show that the membrane alterations develop in parallel with, or even in advance of, demonstrable tolerance in the intact organism. It would be even more convincing if production of these same membrane changes by other means (dietary or other) could produce tolerance in the intact organism that has not yet been exposed chronically to alcohol. During the past year, we have been attempting to do this in collaboration with Clandinin, Beauge, and Rangaraj. However,

this is only preliminary work, and much remains to be done before a definitive answer is possible.

Despite this, the membrane changes described in this section are possibly the best lead to date to the basic inherent adaptive response that forms the cellular basis of tolerance. If this is so, the greatest challenge will be to determine how environmental, behavioral, and neuroendocrine influences modulate the rate and degree of development, and the duration, of these cellular changes. If future investigators can answer that question, they will have made an enormous stride toward the explanation of not only alcohol tolerance but of learning and physiological adaptation that are fundamental to life itself.

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MEMBRANE MECHANISM OF ETHANOL TOLERANCE AND PHYSICAL DEPENDENCE

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Overview

Dora B. Goldstein

The disordering effect of ethanol on membrane lipid bilayers is now well established, and investigators are studying the chronic effects of ethanol on membranes, effects that might be responsible for some aspects of tolerance and physical dependence. Indeed, we now observe that membranes of chronically ethanol-treated mice are relatively resistant to the in vitro disordering effect of alcohol--i.e., the membranes are tolerant. The effect is seen in synaptosomal plasma membranes, erythrocytes, and hepatic membranes. Cross-tolerance to other sedative drugs has also been reported.

Two types of membrane tolerance have been observed, which might be designated "divergent" and "parallel." In the former, the intrinsic or baseline order of the membranes is unaffected by prior treatment with alcohol. The membranes appear normal if no drug is present, but a difference appears when ethanol is added back in vitro. The membranes from ethanol-treated animals are less susceptible to disordering than are membranes from control animals; there is a change in the slope of the concentration-response curve. This type of tolerance could be explained by a change in composition of the membrane so that it resists the entry of drugs, as Rubin and coworkers have reported. In contrast, in the parallel type of tolerance, the membranes from ethanol-treated animals are stiffer than those of controls. On addition of ethanol in vitro these membranes are disordered with the same slope as controls. Since the tolerant membranes start from a higher baseline, they need more ethanol to bring them to a given degree of disorder. This type of response may represent a membrane expression of physical dependence in addition to tolerance, since the membranes have become more ordered, apparently to counteract the ethanol-induced disorder, and they can be returned to normal by adding ethanol in vitro. The parallel type of tolerance cannot be explained by a change in partition coefficient of ethanol. We have not yet identified the conditions that reveal divergent or parallel tolerance. Neither type seems to be restricted to any particular class of membrane, temperature of observation, or position of probe.

These data fit the concept of homeoviscous adaptation--i.e., the organism modifies its cell membrane constituents to maintain an acceptable degree of order or fluidity. This is known to happen in lower organisms in response to changes in environmental

temperature and may also be an appropriate and feasible response to chronic drug exposure in mammals. Changes in the chemical composition of membrane lipids accompany membrane tolerance. The observed changes are not always qualitatively the same in different animal models. The chemistry differs, but the physics may not. Whether it is extra cholesterol or increased proportions of saturated fatty acids in the phospholipids (or both) is a matter to be investigated under different conditions and with different animal models, but the concept of homeoviscous membrane adaptation holds up well in general.

Membrane proteins are now coming into the picture in two ways. Proteins affect the response of the lipid phase to ethanol, as Harris has shown, and the functional proteins are affected in various ways by the change in physical state of the lipid, as Molinoff's data illustrate. However, Gordon's results tell us that structure and function do not always correlate. It is possible to set up conditions where the chemical and physical properties of membranes are altered without changing mitochondrial function. Conversely, ethanol may affect function under conditions where no change in physical or chemical properties of membranes is observed. This will be meat for future discussion and investigation.

Membrane Mechanisms of Alcohol Tolerance and Dependence

R. Adron Harris

INTRODUCTION

The concept that alcohol tolerance and dependence could be due to membrane adaptation is based on studies of temperature adaptation. Sudden changes in temperature of even a few degrees may markedly alter cellular functions and produce detrimental effects. Many organisms can, however, adapt to slow, progressive changes in temperature, thereby maintaining physiological functions and viability despite temperature changes of 20° to 30°C. Temperature adaptation has been demonstrated in many species, including fish, protozoa, and hibernating mammals (Cossins and Prosser 1978; Goldman and Albers 1979; Dickens and Thompson 1980). These organisms alter their membrane lipid composition and membrane physical properties in response to changes in temperature. It is thought that these membrane changes alter the activity of membrane-bound enzymes and ion channels, allowing normal cellular function despite the change in temperature. This has been termed "homeoviscous adaptation" (Sinensky 1974). Acute exposure to ethanol was proposed to act on the membrane lipids in the same manner as temperature, increasing the disorder or "fluidity" of the lipids (Hill and Bangham 1975). Prolonged exposure to alcohol, or a similar intoxicant-anesthetic, would lead to membrane adaptation such that more drug would be required to alter membrane function, resulting in tolerance. Following the analogy to temperature adaptation, chronic alcohol treatment would produce a more rigid membrane. Thus, membrane order would be normal in the presence of ethanol but inappropriately rigid in its absence. It was postulated that such changes could be the basis of tolerance and dependence, respectively (Hill and Bangham 1975).

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Thus, the paradigm of temperature adaptation provided an approach to study ethanol tolerance and dependence. Validation of this approach requires at least three observations: (1) concentrations of ethanol associated with nonlethal intoxication must perturb membrane lipids; (2) chronic administration of ethanol must reduce this membrane perturbation produced by ethanol; and (3) chronic ethanol treatment must increase the rigidity of brain membranes.

Effects of low concentrations of ethanol on brain membranes have been detected by both electron spin resonance (ESR) and fluorescent probes (Chin and Goldstein 1977; Harris and Schroeder 1981, in press). The order parameter of ESR probes and the polarization of fluorescent probes is reduced by ethanol (20 to 100m$\underline{M}$). Both results indicate that ethanol disorders the acyl chains of membrane lipids, and this perturbation is greatest in the hydrophobic core of the membrane (Chin and Goldstein 1981; Harris and Schroeder 1981; Harris and Hitzemann 1981). These studies used synaptic plasma membranes (SPM) prepared from mouse brain. Purified lipids or lipids extracted from brain membranes are also disordered by ethanol, but these preparations are much less sensitive than intact membranes, and it is difficult to obtain appreciable effects with physiologically relevant concentrations of ethanol (Harris and Schroeder 1981). Thus, the first requirement set forth above has been satisfied. Several studies have also addressed the issue of membrane adaptation during chronic ethanol treatment. Chin and Goldstein (1977) used an ESR probe (5-doxylstearate) to test the ethanol sensitivity of brain SPM from mice that were consuming an ethanol-containing liquid diet. They found that chronic alcohol treatment attenuates the membrane-disordering effects of a subsequent in vitro ethanol challenge. Similar results were obtained with liver mitochondria and brain synaptosomes from rats that were ingesting an ethanol-containing liquid diet (Rottenberg et al. 1981). Johnson and coworkers (1979, 1980a,b) treated mice and rats by repeated injection of ethanol and prepared a crude mitochondrial (P_2) fraction from brain. The lipids extracted from these membranes were used to form vesicles (liposomes), which were labeled with a fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene (DPH). Lipid vesicles from ethanol-treated animals were less sensitive to the membrane-perturbing effects than were vesicles from control animals. It should be noted that a high concentration (700m$\underline{M}$) of ethanol was required to disorder these extracted lipids, and the magnitude of the membrane adaptation was small. For example, the polarization of DPH in brain lipids from control rats was decreased by 0.0181 by 700m$\underline{M}$ ethanol, and the same concentration produced a decrease of 0.0168 in lipids from ethanol-dependent rats (Johnson et al. 1980a). The small difference between control and ethanol-treated animals may be because the analyses were conducted with extracted lipids from a mixture of brain membranes. We have prepared brain SPM, mitochondria, and microsomes from mice (DBA/2N) that were drinking an ethanol-containing liquid diet for 7 days. Using DPH as a fluorescent probe of lipid acyl

order, we find that intact SPM showed the greatest adaptation to the disordering effect of ethanol (table 1). Intact SPM from chronically ethanol-treated mice display a clear resistance to the fluidizing effect of ethanol, as compared with pair-fed controls. The adaptation is considerably less for intact microsomal or mitochondrial membranes or for lipids extracted from SPM. These studies demonstrate that chronic alcohol treatment leads to membrane adaptation, resulting in a decreased sensitivity to the membrane-perturbing action of ethanol.

As noted, a striking effect of adaptation to higher temperatures is an increase in membrane rigidity, and a similar effect was postulated as the basis for membrane adaptation to ethanol. This postulate is supported by the results of Rottenberg et al. (1981) and Waring et al. (1981), who demonstrated that liver mitochondrial and brain synaptosomal membranes from ethanol-treated rats are more rigid than those from control rats. Increased rigidity after alcohol treatment has not, however, been a universal finding. The original study of Chin and Goldstein (1977) noted no effect of chronic ethanol on the order of brain synaptosomal, mitochondrial, or myelin membranes or erythrocyte membranes. A subsequent study, using probes located deeper in the membrane, noted an increase in rigidity (Lyon and Goldstein 1982). Using DPH as a fluorescent probe, Johnson and coworkers detected an

TABLE 1.--Membrane adaptation produced by chronic alcohol ingestion

	Decrease in polarization	
Membrane fraction*	Pair-fed control	Chronic ethanol*
Microsomes	0.007±0.001†	0.005±0.002
Mitochondria	0.005±0.001	0.003±0.001
SPM	0.009±0.001	0.004±0.001‡
SPM lipids	0.003±0.001	0.003±0.001

*Membranes were prepared from brain as described by Harris and Schroeder (1981). Male (DBA/2N) mice were given a liquid diet with 5 percent (v/v) ethanol or were pair-fed an isocaloric liquid diet for 7 days.

†Data represent the decrease in fluorescence polarization of DPH produced by in vitro addition of 100m$\underline{M}$ ethanol. Details of the procedure are described by Harris and Schroeder (1981). Values are the mean ±SEM, $\underline{N}$ = 6 to 12.

‡Significantly different from pair-fed control, $\underline{p} < .01$.

increased rigidity of lipids extracted from brain membranes in some experiments (Johnson et al. 1979, 1980b) but not in others (Johnson et al. 1980a). In the experiments described in table 1, we did not find any increased rigidity in the membranes from ethanol-treated mice. Thus, a pronounced attenutation of ethanol effects occured without any apparent changes in membrane rigidity. This result raises the possibility that increased rigidity is not the sole membrane alteration produced by chronic alcohol exposure, and it suggests that the mechanisms responsible for adaptation to alcohol are not identical to those that lead to temperature adaptation.

MECHANISMS OF MEMBRANE ADAPTATION

The results presented in the preceding section demonstrate that brain (and liver) membranes isolated from alcohol tolerant-dependent rodents are resistant to the membrane-disordering effects of ethanol. This adaptation must be due to changes in either membrane composition or the arrangement of membrane components. Studies to date have focused on two components that are important determinants of membrane physical properties: cholesterol and phospholipid acyl saturation. Chronic alcohol treatment increased membrane cholesterol content in some (Chin et al. 1978) but not all (Johnson et al. 1979; Lyon and Goldstein 1982) studies. Experiments in which brain lipids (crude mitochondrial fraction) from chronic ethanol and control animals were reconstituted with varying amounts of cholesterol indicate that the increased cholesterol content produced by ethanol treatment could not be responsible for the observed membrane adaptation (Johnson et al. 1979). In the case of liver mitochondria, the extracted phospholipids display reduced sensitivity to ethanol, although the adaptation may not be as great as is seen with the intact membranes (Waring et al. 1981). These results suggest altered phospholipid composition as one mechanism of membrane adaptation. In liver mitochondria, this postulate is supported by a marked increase in the saturation of acyl groups of cardiolipin (table 2). Cardiolipin is an important component of mitochondrial membranes, but it is not found in SPM or other plasma membranes (Fontaine et al. 1980). In our study of SPM, we found that extracted lipids (phospholipids plus sterols) did not display the ethanol "tolerance" that is found with intact membranes. This result suggests that SPM adaptation may differ from that of mitochondrial adaptation and that the adaptation noted in brain lipids by Johnson et al. (1979, 1980a,b) may be due to mitochondrial lipids. This speculation is supported by the general lack of effect of chronic alcohol treatment on the ratio of unsaturated to saturated (U/S) membrane acyl groups in SPM (table 2). I summarize the acyl composition analyses as U/S ratios because the presence of a single double bond greatly alters the physical properties (e.g., transition temperature) of a lipid, but at physiological temperature, subsequent double bonds have little effect (Stubbs et al. 1981). Studies of temperature adaptation in fish

TABLE 2.--Chronic ethanol treatment and saturation of lipid acyl groups

Species	Treatment	Membrane*	Unsaturated/saturated		Reference
			Control	Ethanol	
Mice	Vapor	Brain P_2			Littleton and John (1977)
	10 days	Total PL	1.14	1.01	N = 10
Guinea pigs	Liquid diet	Brain SPM			Sun and Sun (1979)
	21 days	PC	0.57	0.52	N = 3 to 2
		PE	1.70	2.02	
Rats	Liquid diet	Liver mitochondria			Waring et al. (1981)
	30 to 35	PC	1.20	1.21	N = 5
	days	PE	1.02	1.30	
		CL	13.3	6.09	
Swine	Beer	Brain SPM			Harris et al. (in press)
	3 years	Total PL	1.2	1.2	N = 8 to 9

*Abbreviations: P_2, crude mitochondrial fraction; PL, phospholipids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; CL, cardiolipin; SPM, synaptic plasma membranes.

also suggest differences between mitochondria and synaptosomes. The increase in membrane rigidity is greater in mitochondria than in synaptosomes (Cossins and Prosser 1982), and the U/S ratio of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) decreases in mitochondrial lipids but not in synaptosomal lipids (Selivonchick et al. 1977). Thus, there is no conclusive evidence that changes in acyl saturation are responsible for the adaptation of synaptic membranes to temperature or ethanol. The mechanism of this adaptation remains to be defined.

CONCLUSIONS

During the past few years we have made great strides in understanding the effects of ethanol on brain membranes. We now know that low concentrations of ethanol disorder the core of synaptic membranes and that chronic ethanol exposure results in adaptation to this perturbation. These observations raise several important questions: (1) How do changes in membrane order affect cellular functioning; (2) What are the mechanisms responsible for membrane adaptation to ethanol; and (3) Does the membrane adaptation account for tolerance and dependence? Only now are we beginning to understand enough about both the biochemistry of cell membranes and the pharmacology of ethanol to approach these questions.

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Physical Properties of Brain Membranes During Ethanol Tolerance and Physical Dependence

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Much of the work in our laboratory has been directed toward the chronic effects of ethanol in mice and on their cell membranes. We have been testing the hypothesis that the plasma cell membrane is the site of the action of ethanol and the site of the adaptive changes that might underlie ethanol tolerance and physical dependence. We have used electron paramagnetic resonance (EPR) to monitor the change in physical state of membranes, correlated with behavioral and physiological effects of ethanol.

Alcohols probably act in hydrophobic regions of cell membranes since their anesthetic potencies correlate with their lipid solubilities (Seeman 1972). Drugs in this group are generally assumed to produce their effect in vivo in a nonspecific manner simply by their presence in the membrane. Aberrations in the lipid phase presumably disrupt the function of membrane-bound proteins.

Modern biophysical techniques allow us to examine membrane structure at the molecular level and to test the actions of ethanol on the physical state of brain membranes. With a sensitive EPR technique we measured the effects of ethanol on the order of the membrane bilayer. We used stearic acid derivatives as paramagnetic probes (spin labels). They contain a nitroxide group in an oxazolidine ring (doxyl group). When inserted into membranes, they align their long axes parallel to the fatty acid chains of the phospholipids, and their carboxyl groups remain at the surface near the polar head groups. The EPR signal comes from the unpaired electron in the doxyl group. We have used spin-label probes with the doxyl group attached to the 5th, 12th, or 16th carbon of the acyl chain, thus allowing us to monitor different

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depths of the membrane bilayer. The motion of the nitroxide group was measured by an order parameter (Hubbell and McConnell 1971), which reflected the degree of restraint of the probe by its membrane environment--i.e., the membrane order or membrane fluidity. A computer fit of the relevant peaks of the EPR spectrum increased the sensitivity and accuracy of our measurements so that small changes could be reliably detected.

DISORDERING EFFECTS OF ETHANOL

With 5-doxylstearic acid at 37°C, ethanol in vitro decreases the order parameter of mouse synaptosomal plasma membranes (Chin and Goldstein 1977b). The disordering effect of ethanol was small but was concentration related over the range of 10 to 350m$\underline{M}$. To test whether physical disordering of membrane structure by ethanol was related to the behavioral effects of ethanol, the potencies of a series of aliphatic alcohols were compared in vivo and in vitro. The potency of membrane disordering (using 5-doxylstearic acid) increased with chain length of the alcohol and was correlated not only with membrane solubility but also with an increase in hypnotic potency in vivo, as measured by ED_{50} values for loss of righting reflex in mice (Lyon et al. 1981). More recently, a genetic correlation has been found between in vitro membrane actions (measured with the 5-doxyl probe) and in vivo effects of ethanol. Brain membranes from mice with a high genetic sensitivity to the hypnotic effects of ethanol (long sleep mice) were clearly more disordered by the addition of ethanol than were membranes from ethanol-resistant mice (short sleep mice) (Goldstein et al. 1982). As a confirmation of this finding, we selected mice with high and low ethanol sensitivity from a genetically heterogeneous population. We measured the brain concentration of ethanol at receovery from ataxia as an index of sensitivity. Synaptosomal plasma membranes from the ethanol-sensitive mice were more strongly disordered by ethanol in vitro than were membranes from the ethanol-resistant group. These pharmacological and genetic correlations suggest that membrane disordering may mediate some aspects of ethanol intoxication.

SELECTIVITY OF ETHANOL EFFECT

Ethanol may affect one region of the membrane more than another, depending on the chemical composition of the membrane and its physical state. For example, myelin, which has a high order parameter, is less sensitive to the disordering effect of ethanol than are synaptic plasma membranes (Chin and Goldstein 1977b). Miller and Pang (1976) found that the effects of anesthetic agents on the order of phospholipid model membranes depend on the chemical composition of the system. Low-cholesterol vesicles with low order parameters are more sensitive to the disordering effects of ethanol than are those with high order parameters and high cholesterol content (Chin and Goldstein 1981).

The effects of ethanol are also greater deeply in the membrane than near the surface (Chin and Goldstein 1981). The order of mouse synaptosomal plasma membranes was sampled at different depths with 5-, 12-, or 16-doxylstearic acid at 19°C. As expected, the order parameter was lowest with 16-doxylstearic acid and highest with the 5-doxyl probe, reflecting the known spin-label gradient of increasing acyl chain mobility toward the center of the bilayer (Hubbell and McConnell 1971). The disordering effect of ethanol was greater with 12- or 16-doxylstearic acid than with the 5-doxyl spin label in either brain or phospholipid membranes. This result was unexpected since we assumed that ethanol's hydroxyl group would be held to the outer regions of the membrane by hydrogen bonds and would exert its strongest effect near the surface. Other drugs such as butanol, halothane, or ketamine also produce a larger disordering effect with 16-doxyl than with 5-doxylstearic acid in pig synaptosomal plasma membranes or lipid extracted from them (Lenaz et al. 1979). Our data do not indicate the actual location of the ethanol molecule in the bilayer, but they show where it exerts its greatest disordering effect. We also found that the effects of ethanol were greater at high than at low temperature (Chin and Goldstein 1981). These data show that membrane sensitivity to ethanol varies with the chemical composition, the location of the probe, and the temperature. Ethanol may act more strongly in relatively disordered domains of neuronal membranes. If this is so, differences in membrane composition might mediate differential sensitivity to ethanol, both anatomically and over time.

CHRONIC ETHANOL TREATMENT

Drug tolerance and physical dependence may represent an adaptation to a perturbing drug stimulus. In 1975, Hill and Bangham proposed that the physical properties of neuronal membranes varied with excitability state and that cells might adapt to drugs by changing the composition of their membrane lipids. The appropriate adaptive response to the disordering effect of ethanol would be to restore the fluidity of the membrane toward normal in the presence of ethanol (tolerance). On sudden removal of the drug, the membrane would be abnormally rigid, which might account for the withdrawal hyperexcitability state (physical dependence). This process is analogous to homeoviscous adaptation of lower organisms to changing environmental temperature (Sinensky 1974).

To test this hypothesis, we examined membranes from mice that were chronically treated with ethanol, and we looked for changes in intrinsic order, differential response to ethanol, and altered chemical composition. We administered ethanol in liquid diets or by inhalation. For the liquid-diet regimen, we used an inbred strain of mice (DBA/2J) and fed them for 8 days on a liquid diet containing ethanol as 33 percent of the calories. The control animals were pair-fed the same diet, in which sucrose replaced ethanol isocalorically. Based on alcohol intake, those animals selected for the biophysical studies were definitely dependent

(Goldstein and Arnold 1976). Tolerance was not quantitated in this experiment, but the animals appeared to be tolerant since they did not lose their righting reflex at high blood-alcohol levels (mean of 3.4 mg/ml) that were clearly intoxicating for untreated animals. For the inhalation experiments we used Swiss Webster mice that were treated for 3 days with ethanol by inhalation, a technique that produces physical dependence (Goldstein 1972) and tolerance. Pyrazole was given daily to stabilize the blood-ethanol concentration at about 2 to 2.5 mg/ml. Synaptosomal plasma membranes were prepared when the mice were withdrawn from ethanol. In the liquid-diet experiment, membranes were spin labeled only with 5-doxylstearic acid, but in the inhalation experiment the 12-doxyl and 16-doxyl probes were also used to explore more deeply in the membrane bilayer.

PHYSICAL DEPENDENCE

In the inhalation experiment, with the 12-doxyl probe at 19°C, the order parameter was significantly increased in the membranes from the ethanol-dependent animals compared with those from the pyrazole control mice (Lyon and Goldstein in press). The increased membrane rigidity may represent a membrane component of physical dependence. Chronic ethanol treatment, however, did not change the baseline order parameters with either method of alcohol administration when we used the 5-doxyl probe at 37°C (Chin and Goldstein 1977a; Lyon and Goldstein in press). We also found no evidence of membrane dependence with 5-doxyl- or 16-doxylstearic acid at 19°C. In contrast, Rottenberg et al. (1981) reported increased ordering with the 5-doxyl probe in synaptic membranes from rats on an ethanol liquid diet for 35 days.

TOLERANCE

For both alcohol regimens, membranes from the ethanol-tolerant animals were tolerant to the in vitro disordering effects of ethanol with either the 5-doxyl or 12-doxyl probe (Chin and Goldstein 1977a; Lyon and Goldstein in press). At each concentration of ethanol added in vitro, membranes from the chronically treated mice were less disordered than those from the controls. Two types of membrane-tolerance curves were observed on addition of ethanol in vitro to membranes from mice that were tolerant to ethanol. At 37°C with the 5-doxyl probe the order parameters of the membranes from control and treated mice started at the same initial values in the absence of ethanol, but they diverged as the concentration of added ethanol increased. This divergent type of membrane tolerance was seen in both the liquid diet and the inhalation experiments. In the latter procedure, synaptic membranes were also cross-tolerant to the in vitro addition of tert-butyl alcohol, suggesting a hydrophobic locus of this apparent adaptation. The second type of membrane tolerance was observed at 19°C with the 12-doxyl probe. The alcohol concentration-response curves of control and treated membranes were parallel;

order parameters of the tolerant membranes were higher in the absence and presence of alcohol.

Membrane cross-tolerance to tert-butyl alcohol was also seen as a parallel shift of the concentration-response curve to the right. The two types of tolerance curves seen here in brain membranes are similar to those reported for rat liver mitochondrial membranes (Waring et al. 1981, 1982). Divergent curves were observed in mitochondrial membranes at high temperature (35°C) with both 5-doxyl- and 12-doxylstearic acid; parallel curves were evident at 15°C with the 5-doxyl label. The divergent type of curve, which shows no change in intrinsic order in the absence of ethanol, could be the result of a decrease in the partition coefficient of ethanol in the tolerant membrane (Rottenberg et al. 1981). The parallel type of curve, however, may be due to a change in the physical state of the interior of the membrane. Irrespective of the shape of the curve, tolerance develops to ethanol both in vitro at the membrane level and in vivo in our experiments.

LIPID COMPOSITION

We then looked for biochemical correlates of the physical differences in the membranes. We chose to measure cholesterol content because it is a common component of mammalian membranes and may be important in regulating the fluidity of the bilayer. Cholesterol increases the order of model phospholipid membranes above their phase-transition temperature and offsets the disordering effect of ethanol (Chin and Goldstein 1981). We found that synaptosomal plasma membranes from DBA mice that were fed ethanol in a liquid diet had a higher cholesterol/phospholipid molar ratio than membranes from the pair-fed controls (Chin et al. 1978). The elevated ratio was due to a significant increase in cholesterol with no change in phospholipid. The 10 percent increase in cholesterol (per milligram of protein) in synaptosomal plasma membranes may be meaningful and could partly explain the alterations in the physical properties of the membranes from DBA mice. However, the 3-day ethanol inhalation treatment produced no difference in cholesterol content, phospholipid content, or the cholesterol/phospholipid molar ratio in synaptosomal plasma membranes from Swiss Webster mice. Thus the increased order in membranes from the ethanol-treated mice in the inhalation experiment cannot be attributed to alterations in cholesterol content, and we cannot be certain that the elevated cholesterol in the liquid-diet experiment caused the membrane resistance to ethanol. Cholesterol may decrease the partitioning of ethanol into the membrane as has been shown for pentobarbital (Miller and Yu 1977), or it may somehow dampen its disordering effect. Other lipid components may also be involved (Johnson et al. 1979; Littleton et al. 1980).

The observed differences in lipid changes between our two experiments may depend on the strain of mouse or the mode of ethanol administration. In our experiment both DBA and Swiss Webster

mice were physically dependent on ethanol, although membrane cholesterol was increased only in the DBA animals. In contrast, Littleon et al. (1980) exposed DBA and TO Swiss mice to the same regimen of 10 days of ethanol vapor to produce tolerance and measured the composition of the fatty acids in brain synaptic membranes. In their experiment the saturated fatty acids were unchanged in DBA mice but were increased in the TO Swiss animals. Thus, different strains of mice might use different mechanisms to compensate for a disordering stimulus such as ethanol. DBA mice may respond by changing their membrane cholesterol, and another strain may alter some other lipid component, such as fatty acids, to restore the physical state of the bilayer to normal. The mode of ethanol administration could also be a factor in the differential lipid changes we observed. The exposure of the mice to ethanol is continuous and constant by inhalation, but it is intermittent with the liquid diet, depending on the eating habits and intake of the individual animals. Both control and treated mice lost more body weight (28 percent) with the liquid-diet regimen over 8 days than with the inhalation method over 3 days (10 percent).

SUMMARY AND CONCLUSIONS

The small molecule ethanol may act physically in hydrophobic regions of neuronal membranes. With a sensitive EPR technique we showed that low concentrations of ethanol in vitro disordered brain synaptic membranes near the surface of the bilayer. The disruption of membrane structure by the in vitro addition of ethanol was correlated with the behavioral effects of ethanol in pharmacological and genetic experiments. The hypnotic potencies of a series of short-chain alcohols in mice increased with their membrane-disordering potencies in vitro. In the genetic study, membranes from mice with high sensitivity to the hypnotic effects of ethanol were more strongly disordered by ethanol in vitro than those from alcohol-resistant mice. Ethanol may have some selectivity; the disordering effects were greater in the more fluid domains of the bilayer, such as low cholesterol regions, deeply in the interior of the membrane, or at high temperature. When mice were chronically treated with ethanol, a membrane component of tolerance and of physical dependence was observed. Membranes from DBA or Swiss Webster mice that were given ethanol in a liquid diet or by inhalation were tolerant to the disordering effect of ethanol, as measured by the higher concentration of alcohol required to reduce membrane order to a given disordered state. With the 12-doxyl probe at 19°C, membranes from physically dependent mice were more ordered than those from control animals. With chronic ethanol treatment the cholesterol content was elevated in synaptosomal plasma membranes only from DBA mice on a liquid diet but not from Swiss Webster mice on the inhalation regimen. Since alterations in physical properties of the membranes parallel the behavioral changes associated with acute and chronic ethanol treatment, the data suggest that membrane disordering may be essential for some aspects of ethanol intoxication, tolerance, and physical dependence.

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Effects of Ethanol on Mitochondrial Membranes

William S. Thayer, Hagai Rottenberg, and Emanuel Rubin

INTRODUCTION

Our recent studies have indicated that chronic consumption of ethanol by rats leads to highly specific alterations in the protein composition of the hepatic inner mitochondrial membrane and to changes in the lipid structure of biological membranes (Thayer and Rubin 1979, 1980, 1981, in press; Thayer et al. 1980; Waring et al. 1981, 1982). The former are due to quantitative decreases in the contents of specific proteins within the membrane, rather than to qualitative changes in molecular structure or kinetic properties of individual proteins. Chronic ethanol consumption leads to substantial decreases in the contents of cytochrome oxidase, cytochromes b, iron-sulfur clusters of NADH (nicotinamide adenine dinucleotide) dehydrogenase, and ATPase (adenosine triphosphatase). The contents of other components of the respiratory chain, including cytochromes c plus c_1, ubiquinone, iron-sulfur clusters of succinate dehydrogenase, and flavins, are unaltered after ethanol treatment. The net result of these selective compositional changes is a membrane whose protein structure differs markedly from that of a normal liver mitochondrial membrane. These changes in enzymatically functional membrane components can account for many of the characteristic effects of ethanol consumption on mitochondrial function in vitro.

The alterations of mitochondrial structure and function produced by chronic ethanol consumption have been studied at three distinct levels of membrane organization. These include isolated liver mitochondria, purified hepatic inner mitochondrial membrane vesicles (submitochondrial particles), and intact hepatocytes. Submitochondrial particles provide a simpler experimental system than intact mitochondria because they have an enriched activity of the respiratory-phosphorylation apparatus. They exhibit a membrane sidedness opposite that of intact mitochondria, an orientation that eliminates the need for transport systems and allows

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unrestricted access of substrates and adenine nucleotides to the respiratory-phosphorylation apparatus. Intact hepatocytes provide a more complex experimental system than isolated mitochondria, owing to regulatory interactions imposed by the plasma membrane and other organelles. In addition, studies of mitochondrial function in heptaocytes may reflect physiological conditions more realistically than studies conducted with the isolated organelle. Our experimental studies at these three levels of membrane organization have given consistent results that support the pathophysiological origin of membrane protein alterations induced by chronic ethanol consumption.

CYTOCHROME OXIDASE

A decrease in cytochrome oxidase (aa_3) as a result of chronic ethanol consumption was the earliest alteration of mitochondrial membrane protein content to be reported (Rubin et al. 1970), and it has been noted by several intestigators (Thayer and Rubin 1979, 1981; Koch et al. 1977; Bernstein and Penniall 1978; Schilling and Reitz 1980). In early studies conducted with mitochondria that was isolated from rats that were fed ethanol chronically, a 20 percent decrease in chemically reducible cytochrome aa_3 was observed (Koch et al. 1977). More recent studies of rats that were fed ethanol for a longer period have demonstrated a more substantial decrease in cytochrome aa_3 content. Liver mitochondria, submitochondrial particles, and hepatocytes prepared from rats that were fed ethanol for 40 days all exhibit a 40 to 50 percent decrease in cytochrome aa_3 content (Thayer and Rubin 1981). In each case, our determinations of cytochrome oxidase, as well as other cytochromes, were based on characteristic heme absorbance differences between the reduced and oxidized states, as measured spectrophotometrically. In addition, we studied intact, rather than detergent-solubilized, preparations and used respiratory substrates as physiological reductants. Although the content of cytochrome oxidase is decreased by ethanol consumption, the kinetic properties of the oxidase remaining in the membrane are unchanged (Thayer and Rubin 1980, 1981). The fraction of cytochrome oxidase that remains in the membrane after ethanol treatment interacts with all of the cytochrome c. The content of the latter is not changed by chronic ethanol consumption.

CYTOCHROMES b

Chronic ethanol treatment also causes a decrease of about 50 percent in the cytochromes b content of the membrane (Thayer and Rubin 1981, in press). This finding was determined from the difference in the reduced spectra minus the oxidized spectra, which were recorded aerobically in the presence of the respiratory inhibitor antimycin. These conditions promote reduction of the b cytochromes while cytochromes c_1 plus c, as well as aa_3, remain oxidized. Experimentally, this method allows more accurate measurement of cytochrome b content than if the spectra are recorded

under anaerobiosis or in the presence of dithionite because spectral overlap due to absorbance of reduced cytochromes c plus c_1 does not occur. Spectral overlap seriously limits the quantitation of cytochrome b content. This problem is accentuated with membranes from ethanol-treated rats because the contents of cytochromes c plus c_1 are unchanged; thus, the degree of interference in the measurement of cytochrome b is enhanced with these membranes. For this reason, the extent of the ethanol-induced decrease in cytochrome b content was underestimated in earlier publications because the initial determinations were obtained from spectra of samples that had been reduced chemically with dithionite (Thayer and Rubin 1979).

The alteration of cytochrome b is complex, however, owing to the existence of at least two distinct species of cytochrome b, b-562 and b-566, in the electron-transfer chain. More detailed spectral studies comparing the extent of cytochrome b reduction under substrate-reduced anaerobic conditions with that in the presence of antimycin have suggested that little enzymatically reducible cytochrome b-566 is present in membranes from ethanol-treated rats (Thayer and Rubin 1981). Thus, chronic ethanol consumption appears to decrease the content of cytochrome b-566 more extensively than that of cytochrome b-562.

The decrease in cytochrome b content has been confimed by an alternative approach that is based on antimycin inhibition. Antimycin binds stoichiometrically to a component (possibly cytochrome b) of the cytochrome bc_1 complex, causing inhibition of electron flow in this region of the respiratory chain. Antimycin titrations of respiration with submitochondrial particles, mitochondria, and hepatocytes have all indicated a decrease of approximately 50 percent in the antimycin titer (minimum amount of inhibitor producing maximal inhibition) in preparations derived from ethanol-treated rats compared with those from controls (Thayer and Rubin in press). However, the maximum extent of inhibition in each of these systems was identical to that observed with preparations from control rats. Measurements of antimycin binding to submitochondrial particles by the quenching of antimycin fluorescence confirmed a similar decrease in the number of high-affinity antimycin-binding sites. These results demonstrate that the effect of chronic ethanol consumption on the cytochrome b content of the membrane is similar in magnitude to that on cytochrome aa_3.

CYTOCHROMES c PLUS c_1

Despite the major effects on cytochromes aa_3 and b, the content of cytochromes c and c_1 is unaffected by chronic ethanol consumption. Measurements with both mitochondria and heptocytes showed no change in cytochrome c plus c_1 content. With submitochondrial particles, a decrease of 15 to 20 percent was observed after ethanol treatment. However, this decrease most likely reflects a slightly greater loss of cytochrome c from the membranes during the preparation of submitochondrial particles from ethanol-treated

rats. Interestingly, the entire complement of cytochrome c within the membrane is enzymatically functional and interacts with cytochrome oxidase, even though the content of the latter is only one-half that of control membranes. As a consequence of the differential effects of ethanol consumption on cytochrome c plus c_1 and cytochrome aa_3, the ratio of cytochromes c plus c_1 to cytochrome aa_3 is effectively doubled in membranes from ethanol-treated rats compared with those from controls. In terms of redox interactions of these cytochromes, this change results in maintenance of cytochromes c plus c_1 at a relatively higher level of steady-state reduction during maximal electron flux (Thayer and Rubin 1981).

UBIQUINONE

The membrane content of ubiquinone, determined after extraction from submitochondrial particles, was unchanged by chronic ethanol consumption (Thayer and Rubin 1981). However, this finding cannot exclude a possible alteration in a minor fraction of the total ubiquinone, which may be associated with a specific ubiquinone-binding protein. The total amount of ubiquinone associated with the electron-transfer chain is much greater than that of the cytochromes and other electron carriers, and the bulk of ubiquinone functions as a reducing equivalent pool.

NADH DEHYDROGENASE

Chronic ethanol consumption affects the membrane content of proteins associated with NADH dehydrogenase. This enzyme contains FMN (flavin mononucleotide) and several iron-sulfur clusters, designated by the prefix N-, which function in the transfer of electrons through this region of the respiratory chain. The iron-sulfur clusters of NADH dehydrogenase are paramagnetic in their reduced states. Each exhibits a distinct electron paramagnetic resonance (EPR) spectrum, owing to different molecular structures and magnetic environments. Thus, EPR spectrometry provides a method for estimating the contents of iron-sulfur clusters within membranes. This approach is analogous to the technique of visible absorption spectrophotometry that was used to determine the cytochromes. Comparison of EPR spectra of NADH-reduced submitochondrial particles revealed decreased amplitudes for signals that are characteristic of some of the iron-sulfur clusters of NADH dehydrogenase in preparations from ethanol-fed rats compared with controls. Signals attributable to clusters N-2, N-3, and N-4 were decreased 20 to 30 percent after ethanol treatment (Thayer et al. 1980). However, cluster N-1b seemed to be unaffected by ethanol treatment. Measurements of NADH dehydrogenase activity with exogenous electron acceptors indicated a 46 percent decrease in V_{max} (Thayer et al. 1980), confirming an effect of ethanol consumption on this enyzme. There was no change in acid-extractable flavin (noncovalently bound) after ethanol treatment (Thayer and Rubin 1981). However, since

several flavoproteins are associated with the respiratory chain in liver submitochondrial particles, this determination is not specific for the flavin of NADH dehydrogenase. The decrease in enzyme activity and alteration in EPR spectra are more specific, and they provide evidence for another alteration of mitochondrial membrane proteins induced by ethanol consumption.

SUCCINATE DEHYDROGENASE

In contrast to the effects on NADH dehydrogenase, chronic ethanol treatment does not affect the membrane content of proteins associated with succinate dehydrogenase. This respiratory-chain enzyme contains covalently bound FAD (flavin adenine dinucleotide) and specific iron-sulfur clusters, designated by the prefix S-. EPR spectra of succinate-reduced submitochondrial particles showed no change in amplitude of signals that are attributable to cluster S-1 after ethanol treatment (Thayer et al. 1980). Cluster S-3, which is paramagnetic in the oxidized state, was also unaffected. Activity measurements with exogenous electron acceptors suggested a slight increase in V_{max} values of succinate dehydrogenase after ethanol treatment (Thayer et al. 1980). Measurements of covalently bound flavin by trypsin treatment of submitochondrial particles after acid extraction displayed no difference after ethanol treatment (Thayer and Rubin 1981). In this case, the flavin determination is specific for succinate dehydrogenase since this enzyme is the only mitochondrial flavoprotein known to have a covalently attached flavin prosthetic group. These data indicate that chronic ethanol consumption does not affect the mitochondrial membrane proteins of succinate dehydrogenase.

ATPase

The membrane content of ATPase is also decreased as a result of chronic ethanol treatment. This conclusion is based only on activity measurements, however, rather than on an independent determination of content. Submitochondrial particles and mitochondria from ethanol-treated rats exhibit a decrease of approximately 30 percent in maximal rate of ATP hydrolysis (Thayer and Rubin 1979; Rottenberg et al. 1980). Intrinsic phosphorylation efficiency, as reflected by P/O ratios of submitochondrial particles and $^{32}P_i$-ATP exchange activity, is decreased about 10 to 15 percent. Actual steady-state rates of ATP synthesis during oxidative phosphorylation are decreased 35 to 40 percent with all substrates in submitochondrial particles (Thayer and Rubin 1979). However, with this system the decreased rates of ATP synthesis are due mainly to the decreased contents of specific electron-transfer chain components.

The results of these studies indicate that chronic ethanol consumption leads to substantial alterations in the contents of functional mitochondrial membrane proteins. However, this conclusion is

based on spectrophotometric detection of key prosthetic groups associated with the membrane proteins. Although it is likely that absence of prosthetic groups reflects the absence of the actual proteins, this point has not been rigorously established. More interesting, however, are the molecular mechanisms that are responsible for these alterations.

These and other data from our laboratory indicate that hepatic mitochondrial membranes are substantially different in both protein and lipid composition after chronic ethanol consumption. Although the relationships between the alterations of membrane protein and lipid composition are not yet clear, the observations permit the hypothesis that alterations of membrane assembly may represent a major biological response to chronic ethanol consumption. One possible mechanism might be that adaptive changes in phospholipid composition occur in response to the presence of ethanol, and that this decreases insertion or processing of preproteins of key mitochondrial enzymes, thus leading to decreased membrane content. Alternatively, impairment of the synthesis of key proteins (Burke and Rubin 1979) might alter the binding or exhange of certain phospholipids in the membrane, leading to altered lipid as well as protein composition. Chronic ethanol treatment thus may provide a model that is suitable for unraveling fundamental mechanisms of membrane assembly in a mammalian system.

EFFECTS OF ETHANOL ON TEMPERATURE-DEPENDENT PROPERTIES OF MEMBRANE-BOUND ENZYMES

Ethanol in vitro, at physiologic temperature, decreases the transition temperature of various enzymes in the hepatic mitochondrial inner membrane (Rottenberg et al. 1980). By contrast, the transition temperature of these enzymes is increased after chronic ethanol ingestion. Thus, the addition of ethanol in vitro to membranes from chronically treated animals lowers the transition temperature to a level that is comparable with that of control mitochondria. This suggests that the change in the lipid structure of mitochondrial membranes is an adaptive response to the prolonged presence of a disordering agent, such as ethanol. Similar experiments using hepatic microsomes, with calcium uptake as the parameter studied, provided similar data (Ponnappa et al. 1982).

ORDER PARAMETERS OF MITOCHONDRIAL MEMBRANES

In vitro, ethanol decreases the order parameter of hepatic mitochondrial membranes, as measured with spin labels and electron spin resonance (ESR) (Waring et al. 1981). After chronic ethanol intoxication, mitochondrial membranes show a significant resistance to this fluidizing effect of ethanol. This increased rigidity of the membrane is also evidenced by an increase in the

temperature of the discontinuity when the order parameter is plotted as a function of temperature (Waring et al. 1982). The above changes are also demonstrable in vesicles made from phospholipids that are isolated from the mitochrondria of ethanol-fed animals. In studies of lipid composition, the most pronounced change in mitochondrial membranes after chronic ethanol consumption appears to be an increased saturation of cardiolipin, a negatively charged phospholipid that is unique to mitochondria (Waring et al. 1981).

EFFECT OF CHRONIC ETHANOL CONSUMPTION ON PARTITION COEFFICIENTS OF LIPOPHILIC COMPOUNDS

In general, there is an inverse relationship between molecular order and partition coefficients of lipophilic compounds in biological membranes. This proved to be true in the case of chronic ethanol intoxication. The partition coefficient of 5-nitroxydecane, a spin probe, ethanol, and the inhalation anesthetic, halothane, were all diminished by chronic ethanol treatment (Rottenberg et al. 1981). We have also demonstrated such decreased partitioning in synaptosomal and erythrocyte membranes.

The changes in lipid composition and structural properties of mitochondrial membranes, as noted above, may influence phospholipid and protein assembly in the mitochondrial and other membranes. In addition, the changes in lipid structure also suggest that chronic ethanol intoxication leads to an increased molecular order of biological membranes as an adaptive response to prolonged perturbation of the membranes by ethanol binding. The increased molecular order then results in decreased partition of lipophilic compounds into the membrane. Further work is necessary to elucidate the impact of both injurious and adaptive changes in hepatic mitochondria and other biological membranes.

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Effect of Chronic Ethanol Consumption on Membranes and Associated Functions

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Chronic ethanol ingestion by man or animals leads to morphological and functional changes in the liver (Popper and Lieber 1980; Lieber 1981). The molecular mechanisms underlying these changes, however, are not well defined (Lieber 1981). Recently Rottenberg et al. (1981) proposed that "the chronic consumption of ethanol induces an adaptation of membrane composition causing increased membrane rigidity (decreased fluidity). The increased rigidity impairs normal membrane functions in the absence of ethanol, but in the presence of moderate concentrations of ethanol, the membrane becomes sufficiently fluid to resemble normal membrane (dependence)."

This concept could explain the many diversified and sometimes apparently contradictory effects of ethanol on hepatic functions noted by many investigators, but it moves the speculation to the membranes. Thus, further confirmation seems desirable.

For many years, it has been realized that the functional properties of the membrane are modulated in part by the interaction between membrane-bound proteins and the phospholipids (Singer 1974; Zakim and Vessy 1976). These compounds, in addition to cholesterol and cations, maintain the structural integrity of the membranes. The Arrhenius plots of membrane-bound enzymes from hepatic preparations of ethanol-fed rats have distinctly different patterns from those of the controls, suggesting that alterations have occurred in the membrane's structural properties (Waring et al. 1982). However, these changes have not been directly correlated with alterations in functions of these membranes. In this study we focus on correlating the well-documented changes in hepatic mitochondrial functions with the composition and physical state of this organelle membrane. Two species of animals were tested, the rat, which consumes 36 percent of its caloric intake as ethanol and develops a fatty liver, and the subhuman primate,

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the baboon, which consumes 50 percent of its caloric intake as ethanol and develops the full spectrum of alcoholic liver disease.

METHODS

ANIMALS

Baboon Studies

The baboons used were either Papio hamadryas or olive and yellow baboons. The animals were pair-fed a nutritionally adequate liquid diet containing 50 percent of the total calories either as ethanol or an isocaloric equivalent of carbohydrate for 2 to 7 years (Lieber and DeCarli 1976). In all experiments the ethanol-fed baboons and their pair-fed controls were studied on the same day. All food except water was withdrawn at 4:00 p.m. the day before surgery. Baboons maintained on chow were also used in this study, and the day before surgery food was removed at 4:00 p.m. Surgical biopsies of the liver were obtained under ketamine anesthesia the next morning.

Rat Studies

Male Sprague-Dawley rats were pair-fed a liquid diet containing 36 percent of the caloric content either as ethanol or as an isocalorical equivalent of carbohydrate for 4 to 6 weeks. The constituents of this diet have been described (Lieber and DeCarli 1976). The day before sacrifice, the animals were given one-third the daily ration at 9:00 a.m., and at 4:00 p.m. all food except water was removed. They were sacrificed the following morning between 8:00 and 9:00 a.m.

PREPARATION OF HEPATIC MITOCHONDRIA

Hepatic mitochondria were prepared by differential centrifugation in a medium containing 0.25M sucrose, 10mM Tris-HCl (pH 7.4), and 1mM EDTA (Beattie 1968). The mitochondrial pellet was washed repeatedly. The pellet was then resuspended in 0.25M sucrose (1 ml/g wet weight of liver).

MITOCHONDRIAL RESPIRATION

Oxygen consumption was measured polarographically at 28°C using a Clark oxygen electrode. The capacity of the preparation to oxidize substrates entering site I, II, or III of the respiratory chain was measured as outlined by Estabrook (1967). The final concentration of the substrates was 3.3mM gluatmate, 150μM acetaldehyde, 3.3mM succinate, and 5mM ascorbate plus 0.2mM N,N,N',N'-tetramethyl-p-phenylendiamine. State 3 respiration was induced by the addition of 150μM ADP.

LIPID ANALYSIS OF THE MITOCHONDRIAL MEMBRANES

After isolation, mitochondrial lipids were immediately extracted by the procedure of Bligh and Dyer (1959). Methyl esters of the phospholipid fatty acids were prepared as outlined by Kates (1964) and were analyzed on a gas chromatograph with a 6-foot ×1/8-inch stainless steel column packed with 10 percent butanediole succinate (WHP 80/100 mesh). The temperatures of the injector, columns, and detector were 250°C, 190°C, and 205°C, respectively; the carrier gas was helium, maintained at a flow rate of 75 ml/minute. Peaks were identified by comparison with authentic standards of methyl esters of the fatty acids. The protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

FLUORESCENCE POLARIZATION MEASUREMENTS

Mitochondrial membranes were loaded with fluorescent probes, DL 12 (9-anthroyloxy)stearic acid (12 AS) or 1,6-diphenyl-1,3,5,-hexatriene (DPH) (Brasitus and Schacter 1980). The concentrations of the probes ranged from 1 to 6μM. The final preparation contained membrane equivalent to 75 to 450 μg protein/2.5 ml phosphate-buffered saline (pH 6.6). Steady-state fluorescence polarization measurements were made in a polarization subnanosecond spectrofluorometer at 28°C. The determination of the fluorescence polarization was made with the excitation light set at 368 nm, and the emitted light was detected at 420 nm after passing through an exclusion filter (389 nm). The actual readings of the samples were first corrected for the degree of perturbation due to the presence of the tissue and the suspension media. The results were expressed in terms of fluorescent anisotropy (r_s), according to

$$r_s = \frac{2P}{3-P}$$

where P is the degree of fluorescence polarization obtained from the following formula:

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

where $I_{\parallel}$ and $I_{\perp}$ are intensities of fluorescent light detected through polarizers oriented parallel and perpendicular, respectively, to the excitation light.

RESULTS

In this study the effects of chronic ethanol consumption on hepatic mitochondrial functions and structure were studied in

both ethanol-fed rats and baboons. The capacity of the mitochondria to oxidize substrates entering the respiratory chain was assessed. In mitochondrial preparations from ethanol-fed rats, state 3 respiration was lower by 27.2 percent ($p < .001$) when glutamate was used and 18.3 percent ($p < .001$) when succinate was used as substrate. No significant differences were detected in the capacity of mitochondrial preparations from pair-fed or chow-fed rats to oxidize these substrates. The results with glutamate as substrate are in figure 1. A similar pattern was observed with mitochondrial preparations from the different groups of baboons. However, in this species, the preparations exhibited much more severe dysfunction than those from ethanol-fed rats. State 3 respiration was lower by 59.4, 43.2, 45.1, and 51.1 percent when glutamate, acetaldehyde, succinate, and ascorbate, respectively, were used as substrates, compared with their respective controls (figure 2). When low concentration of ethanol, ranging from 25 to 100mM, were added to the incubation media, the capacity of the mitochondrial preparations from either the ethanol-fed rats or baboons to oxidize substrates was not restored to the control values.

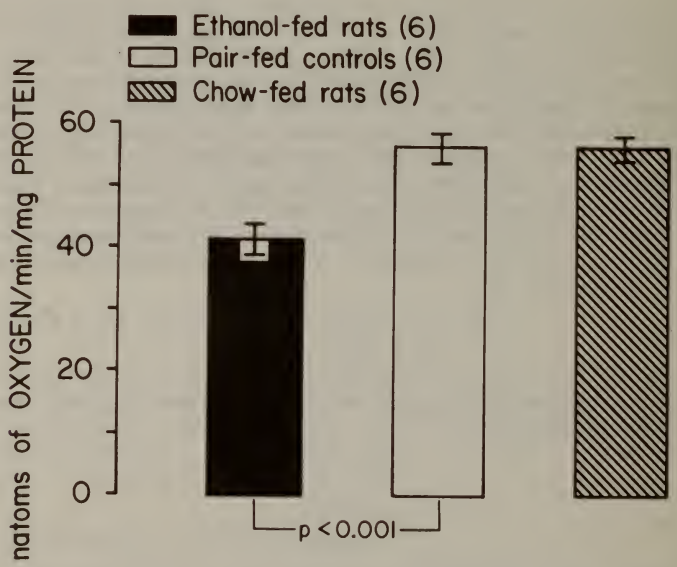

FIGURE 1.--Effect of chronic ethanol consumption on state 3 respiration of rat liver mitochondria. Oxygen consumption was measured polarographically, and results are in nanoatoms of oxygen/milligram of protein. Substrate was glutamate (3.3mM), and state 3 respiration was initiated by the addition of 150μM ADP. Significance was determined by group t-test (compared with pair-fed controls). Number of animals per group is in parentheses.

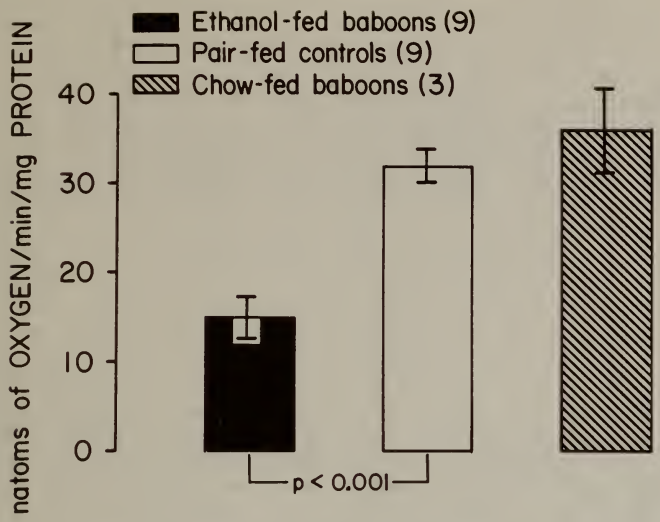

FIGURE 2.--Effect of chronic ethanol consumption on state 3 respiration of baboon liver mitochondria. Oxygen consumption was measured polarographically, and results are in nanoatoms of oxygen/milligram of protein. Substrate was glutamate (3.3mM), and state 3 respiration was initiated by the addition of 150μM ADP. Significance was determined by group t-test compared with pair-fed controls. Number of animals is in parentheses.

The fatty acid composition of the phospholipids of the hepatic mitochondria was determined in all three groups of rats. The phospholipid:protein ratio was similar in both the ethanol-fed rats and their pair-fed controls (0.32±0.04 μmol phospholipid phosphorus/mg protein in the ethanol-fed animal versus 0.30±0.04 in the controls). On the other hand, the preparations from the chow-fed group had significantly lower phospholipid phosphorus per milligram of protein (0.19±0.02 μmol of phospholipid phosphorus) than either experimental group. The fatty acid composition of the phospholipids of mitochondrial preparations is presented in table 1. No significant differences were observed in either the relative or absolute amounts of total saturated, unsaturated, or polyunsaturated fatty acids between the ethanol-fed rats and their pair-fed controls. Indeed, the only differences between these two groups were the relative amounts of the individual saturated fatty acids. There was a greater proportion of stearic acid and less of palmitic acid in the ethanol-fed animals. By contrast, significant differences were noted between the above-mentioned groups and that of the chow-fed animals in the amounts and proportion of the fatty acids of the membrane phospholipids. The relative amounts of unsaturated fatty acids were significantly lower, and the amount of saturated fatty acids was significantly higher in the membranes from chow-fed rats, compared with the other preparations (table 1).

TABLE 1.--Fatty acid composition of the phospholipids of hepatic mitochondrial preparations from ethanol-fed rats, their pair-fed controls, and chow-fed rats after 18 hours of starvation*

Fatty acids (FA), percent by weight	Ethanol-fed rats (8)	Pair-fed controls (8)	Chow-fed rats (10)
16:0	15.0±0.3†	18.2±0.7	19.5±1.0
16:1	0.3±0.0	0.4±0.1	0.6±0.2
16:2	0.3±0.1	0.3±0.1	--
18:0	21.2±0.7†	17.9±0.5	20.6±0.5†
18:1	15.6±0.8	12.5±0.3	9.4±0.4†
18:2	10.9±1.1†	12.7±0.5	18.4±0.5†
20:4w6	26.3±0.8	26.8±0.7	20.3±1.3†
22:5w6	2.5±0.4	2.4±0.3	1.1±0.1†
22:5w3	--	0.3±0.1	--
22:6w3	4.4±0.5	4.0±0.3	5.6±0.3†
Unsaturated FA	59.9±0.9	60.3±0.9	55.6±1.1†
Saturated FA	36.4±0.8	36.5±0.7	40.2±0.8†
Polyunsaturated FA	44.3±1.1	47.0±1.0	45.0±1.3
Micromoles of phospholipid phosphorus/milligram of protein	0.32±0.04	0.30±0.04	0.19±0.01†

*Results are mean ±SEM. Number of animals per group is in parentheses.

† $p < .05$.

† $p < .01$. Significance was determined by group t-test compared with pair-fed controls.

The phospholipid:protein ratio was similar in the mitochondrial preparations from the chow-fed baboons and those that were maintained on ethanol. However, their ratios were significantly lower than those found in the preparations from the pair-fed controls. The fatty acid profiles of these phospholipids is in table 2. No significant differences were detected in the distribution of the total saturated, unsaturated, or polyunsaturated fatty acids in the preparations from the ethanol-fed animals and their pair-fed controls. However, there was a significant increase in the total saturated fatty acids and a decrease in the total unsaturated fatty acid in the phospholipids from mitochondrial preparations of the chow-fed animals. The fatty acid patterns were different in all these groups. The relative amount of 18:2 was significantly greater in ethanol-fed baboons than in the pair-fed controls. On the other hand, the level of arachidonic acid was significantly lower in ethanol-fed baboons than in their controls. Thus, there was a marked decrease in the 20:4w6/18:2w6 ratio in mitochondrial preparations from ethanol-fed baboons when compared with those from the pair-fed controls, but not when compared with those from the chow-fed baboons. It is also of interest that the distribution of the fatty acids of the phospholipids in the membranes of the chow-fed animals was similar to that found in the preparations from the ethanol-fed animals. The major differences were in the relative amounts of palmitic acid, which was significantly greater, and oleic acid, which was significantly lower than the levels detected in the other two groups. The relative amount of arachidonic acid was similar to that found in the ethanol-fed animal, but it was significantly lower than the level found in the pair-fed group. No direct correlation was found between these changes in the fatty acid patterns of the phospholipids and the capacity of the mitochondria from either species to oxidize substrates entering the respiratory chain.

The fluorescence polarization measurements of these preparations from both rats and baboons are in tables 3 and 4. No significant differences were observed in the steady-state fluorescent anisotropy values, as determined with the 12 AS probe, between mitochondrial preparations from ethanol-fed rats and their pair-fed controls (table 3) or ethanol-fed baboons and their pair-fed controls (table 4). The $\bar{r}_s$ (anisotropy) values (mean ±SD) were 0.23±0.05 and 0.23±0.03, respectively, for the ethanol-fed rats and their pair-fed controls, and they were 0.268±0.04 and 0.260 ±0.19, respectively, for the ethanol-fed baboons and their pair-fed controls. The $\bar{r}_s$ values found in the membranes from the chow-fed rats were significantly lower than the membrane values from the rats on a liquid diet (0.19±0.03 versus 0.23±0.05; $p < .05$). The fluidizing effect of ethanol was tested by adding increasing amounts of ethanol in vitro to these preparations (8 and 130mM concentration). This caused a progressive decrease in the microviscosity (increased fluidity) of the membranes. The decrement in $\bar{r}_s$ from 8 to 130mM was highly significant ($\Delta r_s = 0.09$, $p < .01$) in preparations from the experimental rats. By contrast, in membranes from chow-fed rats, increasing concentrations of ethanol affected their microviscosity to a lesser degree (0.19±0.03 to 0.17±0.01).

TABLE 2.--Fatty acid composition of the phospholipids of hepatic mitochondrial preparations from ethanol-fed baboons, their pair-fed controls, and chow-fed baboons after 18 hours of starvation*

Fatty acids (FA), percent by weight	Ethanol-fed baboons (5)	Pair-fed controls (5)	Chow-fed baboons (3)
16:0	16.2±0.8	16.3±0.5	18.1±1.4 †
16:1	0.6±0.2	0.1	0.4±0.05
18:0	21.0±0.8	20.5±1.0	22.3±0.2
18:1	15.4±1.1	13.3±0.9	8.8±0.3†
18:2w6	24.6±1.5†	22.1±0.4	22.4±1.5
20:3w4	2.7±0.3†	1.8±0.5	1.5±0.1
20:4w6	15.0±0.6†	19.9±0.9	14.0±0.4†
22:5w6	1.5±0.3	1.6±0.2	1.3±0.1
22:5w3	0.4	0.7±0.1	--
22:6w3	1.8±0.5	1.5±0.0	6.5±0.5‡
Saturated FA	37.1±0.8	36.9±1.2	40.5±1.4‡
Unsaturated FA	60.9±0.8	60.5±0.6	54.7±1.1‡
Polyunsaturated FA	44.9±1.2	47.4±0.9	46.1±1.1
Micromoles of phospholipid phosphorus/milligram of protein	0.23±0.09‡	0.31±0.04	0.21±0.01†

*Results are mean ±SEM. Number of animals per group is in parentheses. Significance was determined by group t-test compared with pair-fed controls.

†$p < .05$.

‡$p < .001$.

TABLE 3.--Steady-state fluorescence anistropy studies of mitochondrial membranes from ethanol-fed rats, their pair-fed controls, and chow-fed rats*

Source of mitocondrial preparations	Ethanol (m*M*)			
	8	25	100	200
Ethanol-fed rats (7)	.225±.046	.192±.026	.187±.052	.135±.025†
Pair-fed controls (7)	.227±.028	.205±.038	.161±.044	.140±.016†
Chow-fed rats (4)	.195±.021	.199±.010	.162±.022	.162±.010‡

*Results are mean ±SD of r_s (see section on materials and methods) of 12 AS. Number of different preparations is in parentheses. Significance was determined by group *t*-test of the differences in r_s values at 8 and 200m*M* ethanol.

†$p < .01$.

‡$p < .05$.

TABLE 4.--Steady-state fluorescence anistropy values of mitochondrial membranes from ethanol-fed baboons and their pair-fed controls*

Ethanol (m*M*)	12 AS		DPH	
	Ethanol-fed baboons (6)	Pair-fed controls (6)	Ethanol-fed baboons (4)	Pair-fed controls (4)
0	.268±.022	.260±.019	.276±.011	.278±.014
25	.260±.026	.253±.030	.283±.004	.272±.006
100	.242±.024	.240±.024	.254±.007	.257±.004
200	.222±.029†	.230±.025†	.241±.012†	.245±.008†

*Results are mean ±SD of r_s (see section on materials and methods) of 12 AS and DPH. Samples were tested at different ethanol concentrations added in vitro. Number of different preparations tested is in parentheses. Significance was determined by group *t*-test of the difference in r_s values at 0 and 200m*M* ethanol.

†$p < .01$.

The addition of ethanol (25 to 200mM) to the mitochondrial preparations from either the ethanol-fed baboons or their pair-fed controls produced a similar change in the microviscosity. The decrement in r_s was smaller than that observed in preparations from the rat, but the changes were significant [Δr_s = 0.04 for ethanol-fed and 0.03 for the controls (p <.01)].

Similar studies were carried out on the mitochondrial preparations from the baboons, using DPH as the probe. Again, the microviscosity of the membranes from the ethanol-fed baboons and their pair-fed controls was not significantly different; the r_s values were 0.276±0.01 versus 0.278±0.01, respectively. The addition of increasing concentrations of ethanol (25 to 200mM) again caused a decrease in the microviscosity. The changes in r_s were smaller than those observed with 12 AS as a probe, but they were similar in both experimental groups and were significant (p <.01) (table 4).

DISCUSSION

The fact that the phospholipids are required for membrane integrity and partly regulate the activities of the respiratory chain is not disputed here. However, the data from the rats, which were maintained on a diet containing 36 percent of their calories as ethanol for 4 to 5 weeks, and from the baboons, which were maintained on a diet containing 50 percent of their calories as ethanol for 2 to 7 years, do not support the hypothesis that functional changes in the activity of the respiratory chain are necessarily produced by changes in the properties of the membrane phospholipids. Our findings and those of others have demonstrated that ethanol consumption reduced state 3 respiration in hepatic mitochondria of the rat at site 1 (Gordon 1973, Cederbaum et al. 1974; Spach et al. 1979; Thayer and Rubin 1979). In the ethanol-fed baboons, the functions of the respiratory chain were even more severely affected. State 3 respiration was reduced at all three sites of the respiratory chain. On the other hand, no detectable differences were found in the functions of the respiratory chain of hepatic mitochondria from control animals on the liquid diet or chow-fed animals in either species.

However, there were striking differences between the pair-fed controls and the chow-fed animals in both experimental studies, not only in the proportion of the fatty acids of the phospholipids, but also in their relative amounts. The fluid state of the mitochondrial membranes from the chow-fed rats and the pair-fed controls also differed, as did their response when physiological levels of ethanol were added in vitro. The membrane preparations from the chow-fed animals were more resistant to the fluidizing effects of ethanol than the preparations from either of the other groups. This effect may indeed be related to the greater proportion of saturated fatty acids in these membranes. The relative proportions of saturated and unsaturated fatty acids were not significantly altered by the long-term consumption of ethanol in either the rat or the baboon.

The available data on changes in the lipid composition of the mitochondrial membranes from ethanol-fed rats are conflicting (Spach et al. 1979; Thompson and Reitz 1978). The fatty acid content of the diet, the duration of ethanol treatment, the sex of the animal, or the nutritional status of the animal before the experiments could account for these discrepancies. Our findings in the rat agree with those of some investigators (Spach et al. 1979) but disagree with those of others, who have noted a decrease in polyunsaturated fatty acids (Cunningham et al. 1982; Thompson and Reitz 1978; Miceli and Ferrell 1973; French et al. 1971). Our findings in the mitochondrial preparations from the ethanol-fed baboons indicate that there were significant decreases in the amount of arachidonic acid. However, these differences are less than those observed between the two control groups in both species, which exhibited similar functional activities.

This apparent lack of an effect of small changes in the lipid composition of the membrane on the functional activities of the mitochondria has been observed by other investigators who studied factors that regulate the activity of the respiratory chain. The phospholipids of hepatic mitochondrial preparations from rats maintained on essential fatty acid-deficient diets, diets containing 5 percent corn oil and hydrogenated corn oil, had strikingly different fatty acid profiles, which were great enough to produce significant differences in the fluid state of these membranes. However, these differences in physical state of the hydrophobic portion of the membrane were not associated with changes in the capacity of these membrane to phosphorylate ADP (adenosine diphosphate). Not until the level of unsaturated fatty acid had dropped below 40 percent were the functions of the respiratory chain affected (Williams et al. 1972).

Moreover, the degree of saturation of the fatty acids is only one of the many factors that regulate the structural state of the membrane. The effect of other factors, such as the cholesterol and calcium content, and the protein composition of these preparations remain to be defined. Thus, although our results confirm the effect of long-term ethanol feeding on mitochondrial function, a direct causal relationship cannot be established between these changes and those in the structural properties of the membranes related to the phospholipids.

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Activation of Adenylate Cyclase by Ethanol

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INTRODUCTION

Short-chain alcohols have been shown to affect the activity of a number of membrane-bound enzymes. The in vitro addition of ethanol inhibits Na-K-ATPase activity (Sun and Samorajski 1970; Leventhal and Tabakoff 1980). Low concentrations of benzyl alcohol have been reported to stimulate Na-K-ATPase activity, whereas inhibition of enzyme activity was observed when assays were done in the presence of high concentrations of benzyl alcohol (Gordon et al. 1980). The in vitro addition of ethanol has also been shown to inhibit the depolarization-dependent uptake of calcium by synaptosomes (Harris and Hood 1980). Addition of short-chain alcohols has been shown to stimulate adenylate cyclase activity (Gorman and Bitensky 1970; Stock and Schmidt 1978; Rabin and Molinoff 1981). Thus, addition of 2-propanol to hepatic membranes resulted in an increase in basal and glucagon-stimulated, but not epinephrine-stimulated, adenylate cyclase activity (Gorman and Bitensky 1970). In adipocytes, basal, guanyl-5'-yl-imidodiphosphate (GppNHp)-stimulated, and NaF-stimulated activities were increased by 1-propanol, but no effect was observed on hormone-stimulated adenylate cyclase activity (Stock and Schmidt 1978). Ethanol was found to increase both basal and dopamine-stimulated adenylate cyclase activities in striatal membranes (Rabin and Molinoff 1981). The effect of ethanol on hormone-stimulated enzyme activity was greater than its effect on basal activity.

The adenylate cyclase system is a multicomponent system, consisting of a hormone receptor, a regulatory subunit that binds GTP (guanosine triphosphate) and magnesium, and a catalytic subunit (see Johnson et al. 1980). Hormonal stimulation of adenylate cyclase involves binding of the hormone to its receptor, followed by formation of a ternary complex with the regulatory subunit (Kent et al. 1980; Heidenreich et al. 1980). Formation of the ternary complex enhances substitution of GDP by GTP (Cassel

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and Selinger 1978) and decreases the concentration of magnesium required for half-maximal activation of enyzme activity (Clement-Cormier et al. 1978; McSwigan et al. 1980). The binding of GTP to the regulatory subunit causes dissociation of the receptor from the ternary complex (Kent et al. 1980; Heidenreich et al. 1980). Activation of the catalytic subunit involves coupling to an activated regulatory subunit (Ross et al. 1978). Hydrolysis of GTP and GDP returns the system to an inactive state (Cassel and Selinger 1978). The present study was done to determine which components of the adenylate cyclase system are involved in the ethanol-induced stimulation of adenylate cyclase activity.

METHODS

Male mice (C57B1/6J) were killed, and the striata were removed. Tissue was homogenized in 30 volumes of 2m$\underline{M}$ 4-(2-hydroxyethyl)-1-piperazine ethane sulfonate (Hepes) (pH 7.5) containing 2m$\underline{M}$ EGTA. In some studies this crude homogenate was used to measure adenylate cyclase activity. In most studies the homogenate was centrifuged at 20,000$\underline{g}$ for 10 minutes at 4°C, and the resulting pellet was resuspended in Hepes/EGTA buffer. This procedure was repeated three times, and the final pellet was then resuspended in 30 volumes of the same buffer.

S49 lymphoma cells were grown in spinner culture in Dulbecco's modification of Eagle's medium containing 10 percent heat-inactivated horse serum in an atmosphere of 90 percent air and 10 percent carbon dioxide at 37°C. Membranes were prepared as described by Ross et al. (1977).

Adenylate cyclase activity was determined by measuring the conversion of α^{32}P-ATP to α^{32}P-cyclic AMP as previously described (Rabin and Molinoff 1981). A modification (Minneman et al. 1979) of the method of Salomon et al. (1974) was used to isolate the α^{32}P-cyclic AMP.

Data for the activation of adenylate cyclase by GppNHp were analyzed on a CDC 6000 series computer using the FORTRAN subroutine QNMDIF obtained from the Department of Computer Science, Stanford University. This program is a quasi-Newtonian method with difference approximations to derivatives.

RESULTS AND DISCUSSION

The in vitro addition of ethanol caused a dose-related increase in striatal adenylate cyclase activity (figure 1). Basal adenylate cyclase activity was significantly increased by 171m$\underline{M}$ ethanol. The dopamine-stimulated enzyme appeared to be more sensitive to the effects of ethanol since a concentration of 68m$\underline{M}$ ethanol significantly increased adenylate cyclase activity. This activation of adenylate cyclase activity by ethanol was reversible. Adenylate cyclase activity in samples that were pretreated with 300m$\underline{M}$ ethanol

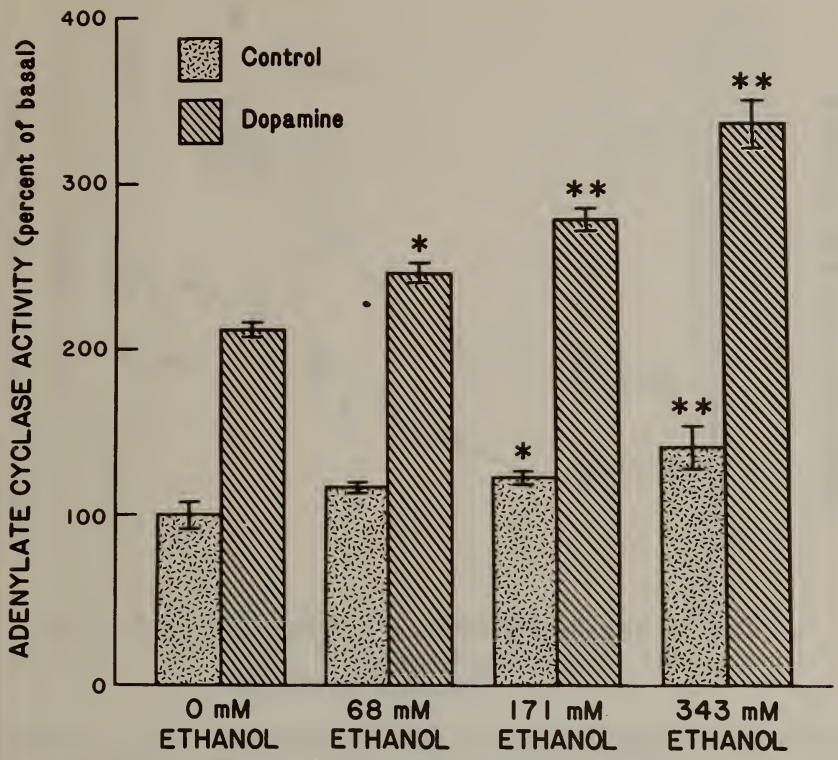

FIGURE 1.--Dose-dependent activation of striatal adenylate cyclase by ethanol. Effect of various doses of ethanol on basal and dopamine-stimulated (100μM) adenylate cyclase activities was determined in a crude homogenate of mouse striatum. Data are percent of the activity measured in the absence of ethanol and dopamine and are plotted as the mean ±SEM for six to eight animals.

*$p < .05$

**$p < .01$ when compared with activity measured in the absence of ethanol (Dunnett's test for multiple comparisons).

and then diluted to a final concentration of 72mM was comparable with the activity in samples exposed to 72mM ethanol directly.

The increase in dopamine-stimulated activity by ethanol was due to both an increase in basal activity and an increase in maximal adenylate cyclase activity (V_{max}) (figure 2). The increase in V_{max} was not entirely due to the change in basal activity since V_{max} minus basal was also increased (42±3.8 pmol/minute/mg in the absence of ethanol compared with 71±5.8 pmol/minute/mg in the

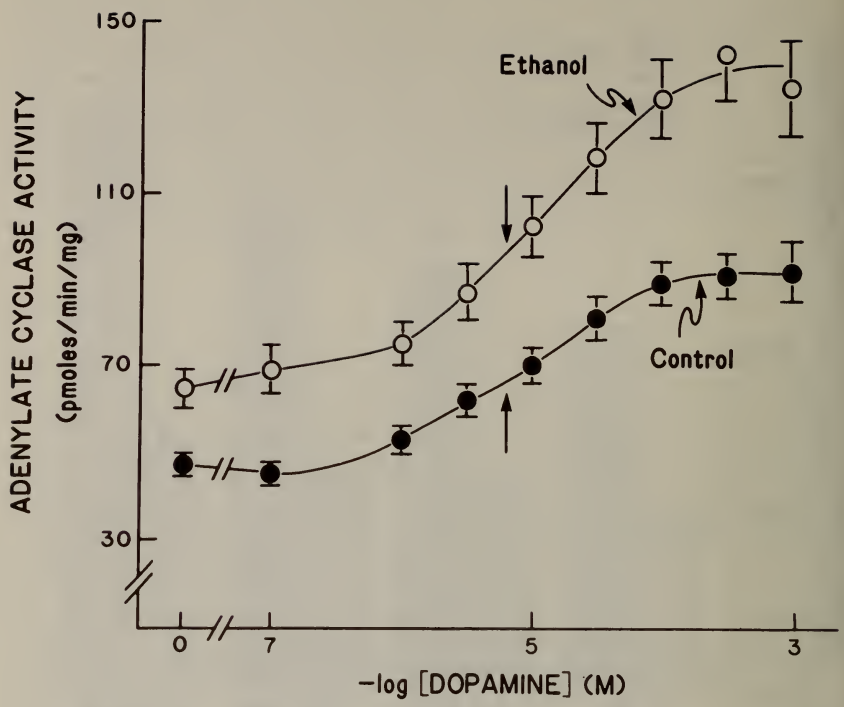

FIGURE 2.--Effect of ethanol on the dose-response curve to dopamine. Crude homogenates of mouse striatum were used to determine the effect of 300m$\underline{M}$ ethanol on the dose-response relationship to dopamine. Data are mean ±SEM values for eight to ten animals. Arrows show the concentration of dopamine required for half-maximal activation.

presence of 300m$\underline{M}$ ethanol; $\underline{p} < .05$). Thus, ethanol appeared to have a synergistic effect on adenylate cyclase activity in the presence of dopamine. The addition of 300m$\underline{M}$ ethanol, however, did not alter the concentration of dopamine required for half-maximal activation of adenylate cyclase activity (figure 2).

Variants of the S49 lympohoma cell line were used to study the effect of ethanol on the various components of the adenylate cyclase system. The S49 wild-type cells contain a β-adrenergic receptor, a catalytic subunit, and a functional regulatory subunit (Ross et al. 1977). Because the CYC^- cells lack a functional regulatory subunit, adenylate cyclase activity is not stimulated by agonists at β-adrenergic receptors (Ross et al. 1978). The catalytic subunit, however, can be directly stimulated by manganese (Ross et al. 1978). In wild-type cells, the addition of ethanol significantly increased adenylate cyclase activity in the

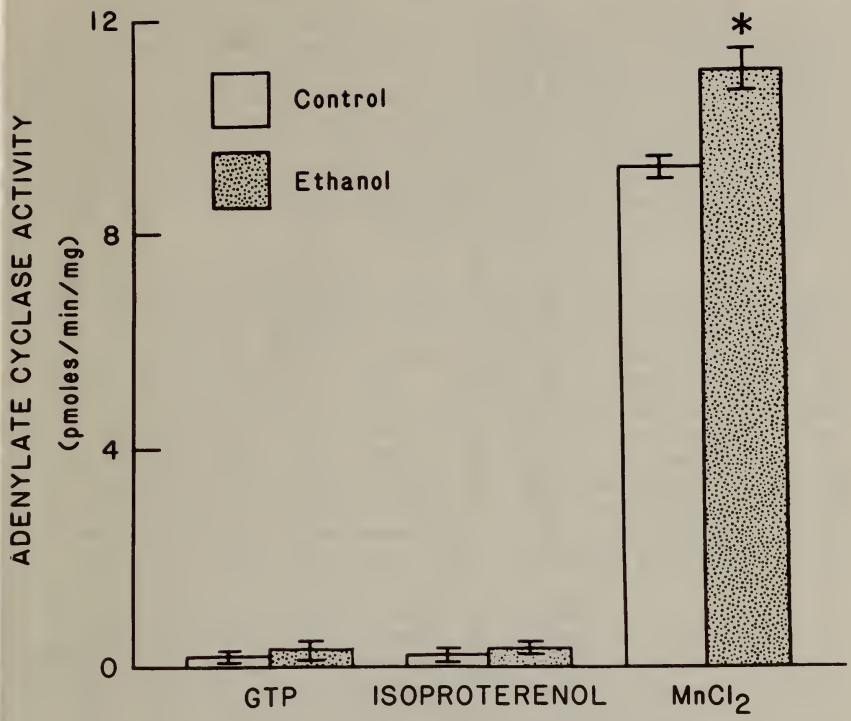

FIGURE 3.--Effect of 400mM ethanol on adenylate cyclase activity in membranes from CYC⁻ cells. Effect was determined in the presence of 50μM GTP, 50μM isoproterenol plus 50μM GTP or 10mM $MnCl_2$. Data are mean ±SEM values for three separate experiments.

*$p < .05$ when compared with activity measured in the absence of ethanol (Student's t-test).

presence of GTP and synergistically increased isoproterenol-stimulated adenylate cyclase activity. The effect of ethanol on adenylate cyclase activity in membranes prepared from CYC⁻ cells is shown in figure 3. Ethanol had no significant effect on adenylate cyclase activity in the presence of GTP or of isoproterenol plus GTP. In the presence of manganese, ethanol significantly increased enyzme activity. These data suggest that at least part of the effect of ethanol on adenylate cyclase involves a direct action on the catalytic subunit. Ethanol did not alter the K_m of the enzyme for the substrate Mg-ATP.

Since ethanol had no effect on adenylate cyclase in the CYC⁻ membranes when assays were carried out in the absence of manganese, it is unlikely that ethanol causes a conversion of the inactive catalytic subunit to an active state. Rather, ethanol

may be acting on the activated form of the enzyme either to increase the turnover number or to extend the half-life of the activated enzyme. Alternatively, ethanol may be facilitating the transition of the inactive enzyme to an activated state by making activation more thermodynamically favorable. Since benzyl alcohol did not stimulate hepatic adenylate cyclase activity after solubilization with Lubrol PX (Gordon et al. 1980), it is reasonable to suggest that the effect of ethanol on the activity of the catalytic subunit is mediated through the annular lipids rather than being a direct effect on the catalytic subunit.

The effects of ethanol on adenylate cyclase activity cannot be explained solely by an action on the catalytic subunit since such an effect would not account for the synergism observed in the presence of hormone. Hormones do not directly affect the catalytic subunit (Ross et al. 1978). Thus, if ethanol were acting exclusively on the catalytic subunit by increasing the turnover number, extending the half-life of the activated enzyme, or facilitating transition to an activated state, then ethanol would be expected to increase basal and hormone-stimulated activities by the same percentage. Since ethanol had a synergistic effect on adenylate cyclase activity in the presence of hormone, it must also be acting at some site other than the catalytic subunit.

The activation of hormone-stimulated adenylate cyclase activity by ethanol does not appear to involve the hormone receptor. Stimulation of adenylate cyclase activity by both NaF and GppNHp, a nonhydrolyzable analog of GTP, requires only the presence of functional regulatory and catalytic subunits (Ross et al. 1978). Since ethanol synergistically increased both NaF- and GppNHp-stimulated adenylate cyclase activities (Stock and Schmidt 1978; Rabin and Molinoff 1982), it is probably acting distal to the hormone receptor.

The possibility that ethanol is acting by inhibiting the GTPase turnoff mechanism has been investigated. If ethanol were acting by inhibiting the turnoff mechanism, the inactivation of GTPase activity before ethanol addition should abolish ethanol's stimulatory effect. Cholera toxin has been shown to activate adenylate cyclase by inhibiting GTPase activity (Cassel and Selinger 1977). Pretreatment of striatal membranes with cholera toxin significantly increased basal and dopamine-stimulated adenylate cyclase activities but did not inhibit subsequent activation by ethanol.

Stimulation of adenylate cyclase by GppNHp occurs after a lag (Londos et al. 1974; Schramm and Rodbell 1975), which is indicative of the rate-limiting step(s) involved in the activation of hysteretic enzymes (Frieden 1979; Neet and Ainslie 1980). This type of hysteretic behavior can be described by the equation:

$$V_t = V_s - (V_s - V_i)e^{-kt}$$

where V_t is activity at time t, V_s is steady-state activity, V_i is initial activity, and k is an apparent rate constant characterizing

TABLE 1.--Effect of ethanol on the activation of striatal adenylate cyclase by 1m*M* guanyl-yl-5'-imidodiphosphate*

	V_s Steady-state activity (pmol/minute/mg)	V_i Initial activity (pmol/minute/mg)	k Apparent rate constant (sec_{-1})
Control	106±7.6 (5)	51±6.6 (5)	2.5±0.35 (6)
300m*M* ethanol	162±2.2 (5)†	77±6.2 (5)‡	3.7±0.37 (6)‡

*Rate of stimulation of adenylate cyclase by 1μ*M* Gpp(NH)p was determined in the presence and absence of 300m*M* ethanol. Tissue was preincubated for 3 minutes at 30°C, and the reaction was initiated by the addition of a prewarmed solution of ATP and Gpp(NH)p.

†$p < .001$ compared with control (Students *t*-test).

‡$p < .05$ compared with control (Students *t*-test).

the transition of V_i to V_s. (Frieden 1979; Neet and Ainslie 1980). The addition of 300mM ethanol increased both V_s and V_i by approximately 50 percent (table 1). This increase in activity was accompanied by a 50 percent increase in the apparent rate constant for activation. These data suggest that ethanol is acting on one of the slow steps in the activation process.

The exchange of GTP for GDP on the regulatory subunit has been suggested as the rate-limiting step in the activation of adenylate cyclase (Blume and Foster 1976; Cassel and Selinger 1978). If ethanol were acting either to increase the dissociation of GDP from the regulatory subunit or to enhance binding of GTP, then the concentration of GTP required for half-maximal activation should be decreased in the presence of ethanol. Addition of GTP to striatal membranes resulted in a dose-dependent increase in dopamine-stimulated adenylate cyclase activity. Although ethanol increased V_{max} minus basal by 50 percent, it did not change the EC_{50} value for GTP. Thus, it appears that ethanol is not acting to increase the turnover of GTP on the regulatory subunit.

In addition to GTP, divalent cations such as magnesium are required for adenylate cyclase activity (Garbers and Johnson 1975). If ethanol were acting to increase the affinity of magnesium for its binding site, then it would be expected to decrease the EC_{50} value for magnesium. Addition of magnesium caused a dose-related increase in adenylate cyclase activity. Under basal conditions, ethanol increased the V_{max} by 60 percent without changing the EC_{50} value for magnesium. Similarly, in the presence of dopamine, ethanol increased the V_{max} without altering the EC_{50} value for magnesium. Thus, ethanol does not appear to be activating adenylate cyclase activity by increasing the affinity of magnesium for its binding site. The present study suggests that the activation of adenylate cyclase by ethanol involves both the catalytic and regulatory subunits. The precise effect of ethanol on these subunits is unclear at present.

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